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PRINCIPAL INVESTIGATOR: Daniel E. Epner, M.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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Daniel E. Epner, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, TX 77030

E-Mail: depner@bcm.tmc.edu

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13. ABSTRACT (Maximum 200 Words)

Many studies have shown that methionine restriction inhibits growth of a variety of human tumor xenografts, including prostate cancers. In contrast, methionine restriction is relatively well tolerated by normal host tissues. The overall goal of the current project is to clarify the molecular mechanisms by which methionine restriction inhibits tumor growth. During the second year of support, we focused on Specific Aim 3, which is to determine whether methionine restriction leads to DNA demethylation in cancer cells. We used Southern blot analysis with methylation-sensitive restriction enzymes, western blot analysis, and RT-PCR to determine whether methionine restriction restored expression of growth inhibitory genes known to be transcriptionally silenced in cancer cells. We studied human prostate, colon, bladder, and leukemia cell lines. Treatment with the demethylating drug 5-azactydine was used as a positive control for DNA demethylation. We found that methionine restriction did not lead to DNA demethylation or re-expression of the genes studied. These results are consistent with published studies showing that DNA demethylation requires cell division, which rapidly ceases in response to methionine restriction. Future studies will focus on the possible role of methionine restriction in regulation of other critical methyl acceptors, such as RNA and protein.

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1) **Introduction**

1. Many animal studies have shown that dietary methionine restriction inhibits growth of a variety of human tumor xenografts, including prostate cancers. In contrast, methionine restriction is well tolerated by normal host tissues for prolonged periods. Recent cell culture studies carried out in the principal investigator's laboratory have defined some of the molecular mechanisms by which methionine restriction inhibits prostate cancer cell growth. On the basis of these preclinical observations, we **hypothesize** that dietary methionine restriction exerts a tumor specific growth inhibitory effect while having minimal deleterious effects on normal tissues.
2. As a preliminary test of this hypothesis, we completed a phase I clinical trial of dietary methionine restriction for 12 adults with refractory solid tumors at the Houston VA Medical Center [1]. Participants received no cancer treatments other than the dietary modification. Dietary methionine restriction was well tolerated for several weeks and reduced plasma methionine levels from 21.6 ± 7.3 μM to 9 ± 4 μM within two weeks, representing a 58% decline. The levels achieved were below the concentration that suppresses tumor cell growth in vitro. In addition, two patients experienced objective responses. One elderly patient with hormone independent prostate cancer exhibited a >25% fall in prostate specific antigen concentration. Although preliminary, these results strongly suggest that dietary methionine restriction is active against hormone independent prostate cancer.
3. The overall goal of the current project is to clarify the molecular mechanisms by which dietary methionine restriction inhibits tumor growth. Methionine is the major methyl donor for methylation of DNA, RNA, protein, and other molecules. DNA methylation is an epigenetic mechanism by which many growth inhibitory and pro-apoptotic genes become transcriptionally silenced in tumors. [2] Reversal of DNA hypermethylation may therefore restore the tumor-suppressive function of these genes and provide a novel approach to cancer therapy. Two demethylating drugs, 5-azacytidine and 5-aza-deoxycytidine, are currently being tested in clinical trials, and several others are in preclinical development.

2) **Body**

1. Specific Aim 2: Determine whether methionine restriction increases oxidative DNA damage in prostate cancer cells.
    i) We employed a genome-wide DNA strand break assay to address this aim. DNA was first denatured by exposure to 100°C for 5 min and then immediately cooled on ice. The reaction mixture included 0.25 μg heat-denatured DNA, 10 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 0.05 mM each dGTP, dTTP, dATP, 0.6 μM dCTP, 7.5 mM DTT, 0.5 unit Klenow polymerase, 0.5 μl [^{32}P]dCTP (3000 Ci/mmol) in a total volume of 25 μl. After incubation for 30 min at 16°C, the reaction was stopped by the addition of an equal volume of 12.5 mM EDTA. The reaction was purified by Micro Bio-Spin P-30 column (Bio-Rad) to remove unincorporated precursors. The radioactivity remaining in the elute was measured in a liquid scintillation counter. The data were expressed as pmols dCTP incorporation/μg DNA.
    ii) We found that methionine restriction led to DNA fragmentation, but only after three or more days, rather than within 24-48 hours (Figure 1). These results suggest that DNA fragmentation was not an *initiator* of cell death, as originally hypothesized, but merely a *reflection* of cell death. If DNA fragmentation were an initiator of cell death in response to methionine restriction, it would precede cell cycle arrest, which occurs within 18-24 hours.

2. Specific Aim 3: Determine whether methionine restriction reduces global DNA methylation or relaxes cancer cell chromatin structure.
   i) We hypothesized that one mechanism by which methionine restriction inhibits prostate cancer cell growth is by demethylating, and thereby transcriptionally activating, growth inhibitory genes. We first used genomic Southern analysis with methylation-sensitive and –insensitive restriction enzymes to test this hypothesis. Genomic DNA was isolated from cells according to a phenol-free protocol (Diagen, Chatsworth, CA). After restriction enzyme digestion, 20 ug of DNA from each sample were fractionated on a 1% agarose gel and then transferred onto a nitrocellular filter. Hybridization was performed using the Quikhyb hybridization solution according to the manufacture's recommendations (Strategene)
   ii) We used the tissue transglutaminase gene as a positive control, since it is known to be transcriptionally regulated by methylation [3]. As expected, we found that treatment of Hela cells with the demethylating drug 5-azacytidine for 2-5 days increased digestion of the tissue transglutaminase gene by HpaII, an enzyme that cuts only demethylated CpG islands [3] (Figure 2). In contrast, growth of cells in methionine-free medium had no such effect, counter to our original hypothesis (not shown).
   iii) Levels of p21, a growth inhibitory cell cycle regulatory protein, are known to increase in PC-3 prostate cancer cells in response to methionine restriction [4]. One mechanism by which p21 levels may increase is by transcriptional activation of the p21 gene. We therefore used the same Southern blot approach as noted above to determine whether the p21 promoter becomes demethylated in response to methionine restriction. However, in this case, neither 5-azacytidine (figure 3) nor methionine restriction (not shown) demethylated the p21 gene in Hela cells. These results are consistent with the possibility that the growth inhibitory activity of methionine restriction on prostate cancer cells is *not* due to demethylation and commensurate transcriptional activation of growth inhibitory and pro-apoptotic genes, as originally hypothesized. However, these few pilot studies do not rule out this possibility.
   iv) In addition to assessing DNA methylation by Southern blot analysis with methylation specific restriction enzymes, as described above, we also used western blot analysis to determine whether methionine restriction increased levels of p16, a growth inhibitory protein known to be transcriptionally silenced in many cancer cell lines and tissues as a result of promoter methylation [5]. We first studied T24 bladder cancer cells, which do not express p16 at baseline but re-express p16, as measured by RT-PCR, following 5-azacytidine treatment [6]. We found that neither methionine restriction nor 5-azacytidine treatment increased p16 levels, as hypothesized (not shown). The observed lack of p16 protein accumulation may reflect the insensitivity of western blot analysis relative to RT-PCR for assessing gene transcriptional activation. The negative results may also simply indicate that methionine restriction did not activate p16 gene transcription, as originally hypothesized. Futher experiments will be required to clarify these issues.
   v) We also used RT-PCR to determine whether methionine restriction restored expression of p16 or hMLH1. hMLH1 is a DNA mismatch repair gene which, like p16, is known to be extensively methylated and therefore transcriptionally silenced in several cancer cell lines [7]. We studied RKO human colon carcinoma and Raji human leukemia cell lines, since both are known to have extensively methylated genomes [8].

We treated cells with the demethylating drug 5-azacytidine for 2-5 days as a positive control. We found that in most experiments 5-azacytidine restored expression of p16 and hMLH, as expected. In contrast, methionine restriction had no such effect (not shown). These results again suggest that methionine restriction does not restore expression of heavily methylated, and therefore transcriptionally silenced growth inhibitory genes, as hypothesized.

vi) In retrospect, the above negative results are consistent with what is known about regulation of DNA methylation. Demethylases, or enzymes that actively remove methyl groups from DNA, have not been identified in mammalian cells and are therefore thought not to exist with possible rare exceptions [9]. DNA demethylation, such as occurs following 5-azacytidine treatment, results from passive attrition, or "wash out" of methyl groups. In other words, methyl groups are not so much removed from DNA as they are simply not added to nascent DNA strands in dividing cells. DNA demethylation requires cell division, which ceases within 18-24 hours in cells deprived of methionine. In retrospect, it is therefore not surprising that our results indicate that methionine restriction does not demethylate DNA in cancer cells. In contrast, the demethylating drug 5-azacytidine does not inhibit cell division for several days, during which time cells synthesize nascent DNA strands lacking methyl groups.

3. Future directions

i) Although DNA methylation is currently a major focus of cancer research, methionine is a methyl donor for several other classes of molecules, such as protein and RNA. We therefore hypothesized that methionine restriction would reduce methylation of molecules other than DNA. To address this hypothesis, we first performed a pilot experiment to measure the relative flux of methyl groups to various pools of molecules, including DNA, RNA, and protein, in cancer cells cultured under control conditions.

ii) To do so, we used a ^{3}H-SAM metabolic labeling assay. $4x10^{5}$ PC3 human prostate cancer cells were seeded in 6 well plates, 0.5 uM ^{3}H-SAM was added to each well, and the cells were cultured for 24 hours. Total cell protein was prepared using the lysis Buffer (20 mM Tris/HCl, pH 8.0; 137 mM NaCl; 10 % w/v glycerol; 10 mM NaF; 1% Triton X-100; 1 mM Na_3VO_4; 2 mM EDTA; 1 mM PMSF; 20 μM leupeptin; and 0.15 units/mL aprotinin). RNA was prepared using Ultrospec RNA isolation reagent (Biotecx Laboratories, Inc.). Genomic DNA was isolated using a kit purchased from Qiangen (Chatsworth, CA). ^{3}H incorporated in the protein, RNA, and DNA from each sample was quantitated by liquid scintilation counter.

iii) We found that protein methylation accounted for greater than 90% of total cell methylation in prostate cancer cells (Figure 4). We also tested the effect of methionine restriction on in vitro methylation of proteins isolated from cancer cells. In this assay, the greater the in vitro methylation, the more demethylated, and therefore "receptive", the protein. We found that methionine restriction of PC-3 prostate cancer cells for just 24 hours led to a 20-fold increase in "methyl receptiveness" of total cellular protein (Figure 5), suggesting that methionine restriction quickly and dramatically reduced protein methylation. Based on these preliminary results, protein methylation appears to be at least an order of magnitude greater quantitatively than DNA methylation, which suggests that protein

demethylation may underlie the selective growth inhibitory effects of methionine restriction in cancer cells.

iv) Future experiments will determine whether methionine restriction inhibits prostate cancer cell growth as a result of protein and/or RNA demethylation, neither of which requires cell division, as DNA demethylation does.

3) **Key Research Accomplishments**: During the second year of support, we focused on Specific Aim 3. In summary, our preliminary results indicate that methionine restriction does not lead to DNA demethylation with commensurate re-expression of transcriptionally silenced genes. These results are consistent with published studies and suggest that methionine restriction may instead affect methylation of other critical molecules, such as RNA and protein.

4) **Reportable Outcomes (publications resulting from DOD support)**

1. Lu, S, Hoestje, SM, Choo, E, **Epner, DE.** Methionine restriction induces apoptosis in prostate cancer via the c-Jun N-terminal kinase-mediated signaling pathway. 2002, Cancer Letters, 179:51.-8.
2. Lu, S, Hoestje, SM, Choo, E, **Epner, DE.** Induction of Caspase-Dependent and -Independent Apoptosis in Response to Methionine Restriction. 2003
3. Li, M, Ittmann, MM, Rowley, DR, Knowlton, AA, Epner, DE. Glutathione S-transferase pi is up regulated in the stromal compartment of hormone independent prostate cancer. 2003, Prostate, 56:98.-105.
4. Lu, S, Chen, GL, Ren, C, **Epner, DE.** Methionine restriction selectively targets thymidylate synthase in prostate cancer cells. Under revision, Biochemical Pharmacology.

5) **Conclusions:** Methionine restriction does not significantly affect DNA methylation in cancer cells but may lead to demethylation of other critical molecules, leading to cell cycle arrest. Future experiments will test this possibility

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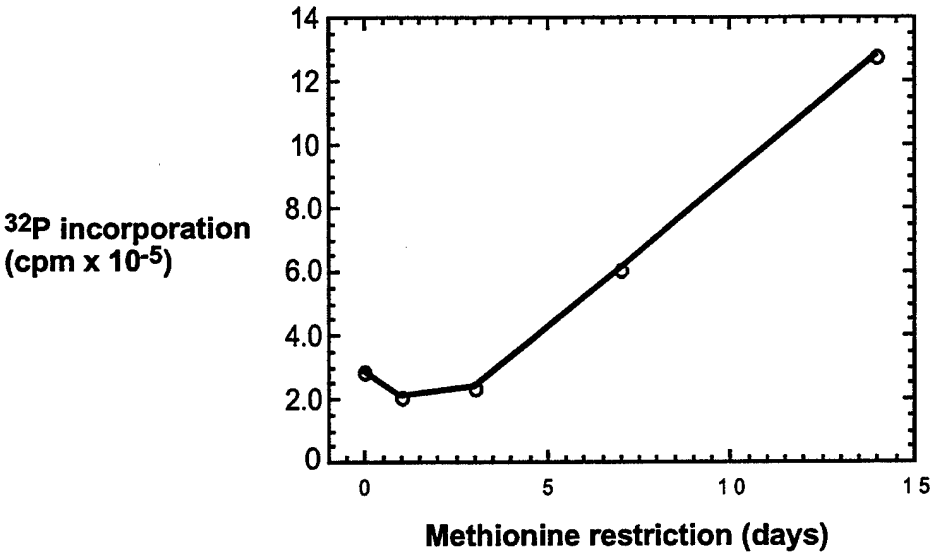

Fig. 1. DNA strand break in response to methionine restriction in PC3 cells. PC3 cells were treated with methionine restriction for 1, 3, 7, and 14 days. Then, genomic DNAs were isolated for DNA strand break assay.

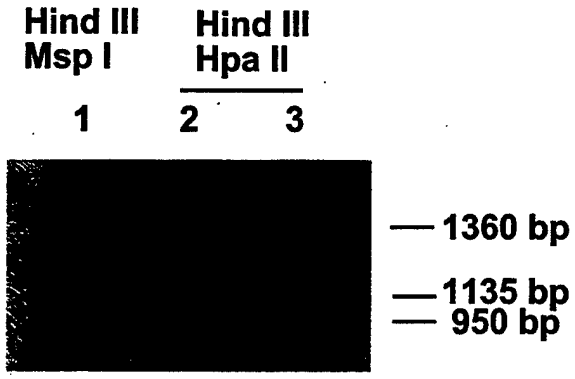

Fig. 2 Methylation level of the human tissue transglutaminase gene promoter in response to 5'-AzaC in Hela cells determined by genomic southern hybridization. Lane 1 and 2, genomic DNA from control Hela cells. Lane 3, DNA from Hela cells treated with 5'-AzaC for 6 days.

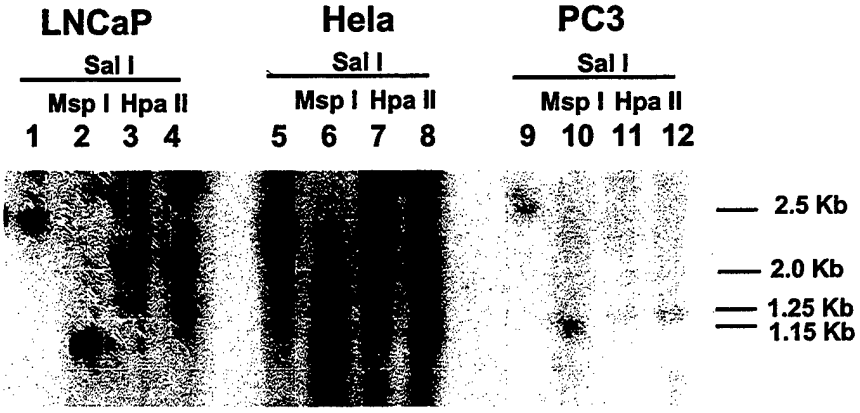

Fig. 3. Methylation level of the human p21 gene promoter in response to 5'-AzaC determined by genomic southern hybridization. Lanes 4, 8, and 12 are DNAs from cells treated with 5'-AzaC for 6 days. The rest lanes are samples from control cells.

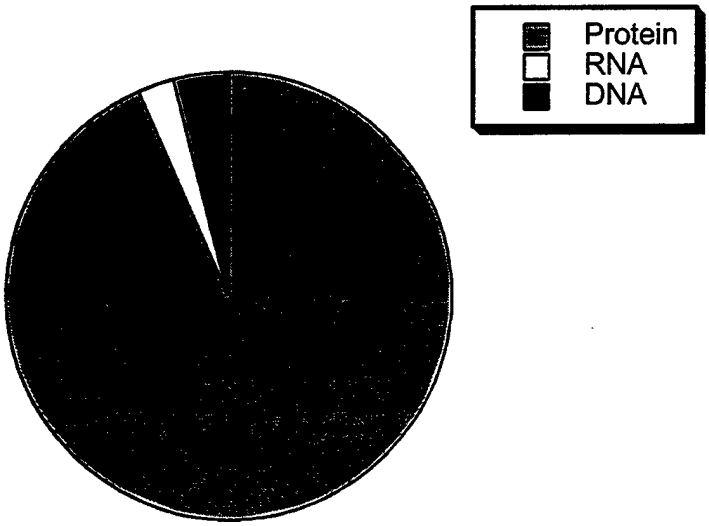

Fig. 4. Distribution of ^{3}H-methyl group among protein, RNA, and DNA determined by metabolic labeling of PC3 cells with ^{3}H-SAM.

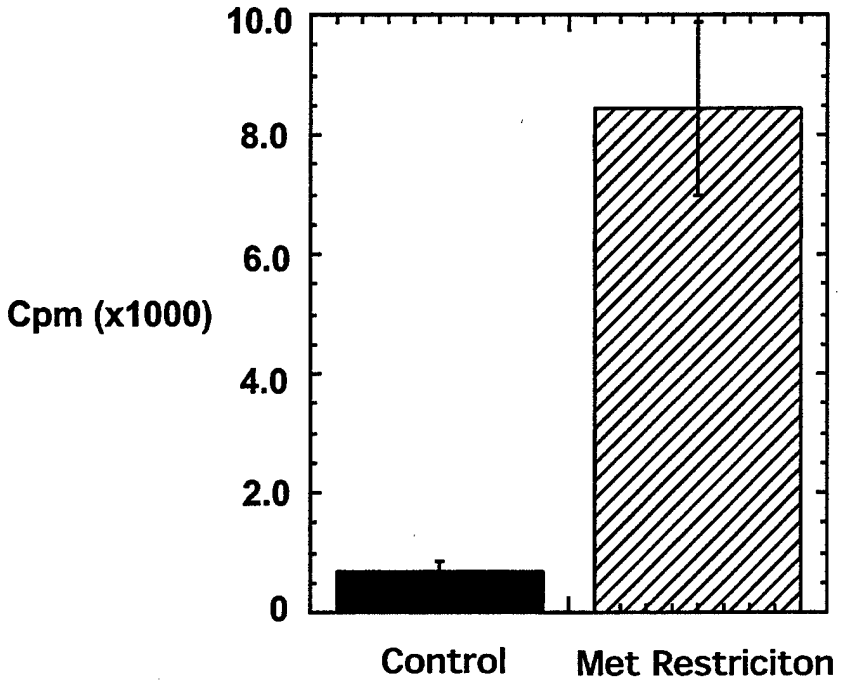

Fig. 5. *In vitro* methylation level of total cell protein in response to methionine restriction for one day in PC3 cells.

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Methionine restriction induces apoptosis of prostate cancer cells via the c-Jun N-terminal kinase-mediated signaling pathway

Shan Lu, Sara M. Hoestje, Eugene M. Choo, Daniel E. Epner*

Department of Medicine, Baylor College of Medicine, and Houston VA Medical Center, Medical Service (111H), 2002 Holcombe Boulevard, Houston, TX 77030, USA

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Abstract

Tumors are relatively more sensitive to methionine restriction than corresponding normal tissues, a phenomenon known as methionine auxotrophy. The current studies were undertaken to elucidate the molecular mechanisms for methionine auxotrophy of prostate cancer cells. We found that the activity of c-Jun N-terminal kinase 1 (JNK1) increased dramatically in response to methionine restriction. Over expression of wild type JNK1 by transient transfection enhanced apoptosis in response to methionine restriction, whereas over expression of a kinase inactive mutant of JNK1 protected PC-3 human prostate cancer cells from apoptosis. We conclude that JNK1 plays a critical role in signaling cancer cells to undergo apoptosis in response to methionine restriction. Published by Elsevier Science Ireland Ltd.

Keywords: Methionine; Neoplasms; Prostate; Signal transduction; Apoptosis

1. Introduction

Methionine is an essential amino acid that cannot be synthesized in mammalian tissues from any of the other standard amino acids. Nonetheless, normal mammalian cells proliferate normally in the absence of methionine as long as homocysteine is present in the growth medium [1], and animals fed diets in which methionine has been replaced by homocysteine suffer no ill effects and grow normally [2,3]. Homocysteine is a non-standard amino acid that has the same structure as methionine except that it lacks a methyl group. Methionine independence of normal tissues is due to remethylation of homocysteine to methionine by two methionine synthases: 5-methyltetrahydrofolate homocysteine methyltransferase and betaine-homocysteine methyltransferase. Function of these enzymes is impaired in some tumors [4–6], whereas it is elevated in several others [7–10]. Regardless of whether methionine synthase activity is impaired or not, most tumors are dependent upon exogenous, preformed methionine and therefore fail to grow even in the presence of homocyteine [11–14]. Dietary methionine restriction causes regression of animal tumors, including human prostate cancer xenografts in nude mice [15,16] and inhibits metastasis in animal models [3,17]. Methioninase, an enzyme that degrades methionine and homocysteine, also inhibits growth of solid tumors and leukemia in animals [18–22]. One clinical trial of dietary methionine restriction combined with chemotherapy showed preliminary evidence of activity against gastric cancer [23]. The selective antitumor activity of methionine restriction

* Corresponding author. Tel.: +1-713-794-7980; fax: +1-713-794-7938.

E-mail address: depner@bcm.tmc.edu (D.E. Epner).

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is not due to an *absolute* difference between benign and malignant tissues, since neither can survive indefinitely in the *complete* absence of endogenous and exogenous methionine. Rather, tumors are *relatively* more sensitive to methionine restriction than normal tissues are, just as many tumors are relatively more sensitive to chemotherapy and radiation therapy.

The body uses a relatively small proportion of dietary methionine for tissue protein synthesis. Most is used for three other specialized functions. First, methionine functions as a methyl donor for methylation of DNA and other molecules. DNA methylation is one of the mechanisms by which gene expression [24] and chromatin structure [25] are regulated. Several growth inhibitory genes are silenced in tumors as a result of hypermethylation [26]. Second, methionine functions in the liver as a sulfur donor for cysteine synthesis, which is critical for glutathione homeostasis. Glutathione (γ-glutamylcysteinylglycine) is a tripeptide that reduces reactive oxygen species, thereby protecting cells from oxidative stress [27]. Third, methionine functions as an aminopropyl donor for synthesis of polyamines, which have far-ranging effects on nuclear structure and cell division [28].

Despite the fact that much is known regarding the specialized functions of methionine, the molecular mechanisms for the selective methionine dependence of tumors remain unclear. Methionine restriction has a cytostatic effect on prostate cancer cells at early stage, causing those cells that lack an intact G1/S checkpoint to arrest predominantly in the G2/M phase of the cell cycle [15,29]. Eventually, methionine restriction induces apoptosis. Apoptosis occurs through either mitochondria-dependent or mitochondria-independent pathways [30]. Mitochondria-dependent apoptosis is generally initiated by environmental and genotoxic stresses, such as ultraviolet or γ-irradiation, DNA damaging drugs, microtubule-damaging drugs, oxidative stress, and osmotic shock [31–34]. Recent studies have demonstrated that activation of the MKKK-MKK-MAPK (mitogen-activated protein kinase) sequential kinase pathway is involved in mitochondria-dependent apoptosis [35–37]. For instance, c-Jun N-terminal kinase 1 (JNK1, also named *s*tress-*a*ctivated *p*rotein *k*inase, SAPK), a member of MAPK family proteins, is activated by ultraviolet or γ-irradiation, oxidative stress, microtubule-damaging drugs, osmotic shock, and other stresses [37]. In addition, over expression of JNK1 induces apoptosis, whereas a kinase inactive mutant of JNK1 blocks cell death in response to ultraviolet C and γ-irradiation in Jurkat T cells and 293T kidney cells [38]. Signaling via this kinase cascade leads to cytochrome c release from mitochondria and subsequent apoptosis [39]. We undertook the present studies to determine whether JNK1 plays a critical role in apoptosis of prostate cancer cells in response to methionine restriction.

2. Materials and methods

2.1. Cell culture

Human metastatic prostate adenocarcinoma cell line PC-3 and Hela human cervical carcinoma cells (American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI-1640 (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) at 37°C in 5% CO_2. Methionine restriction experiments were performed in methionine-free RPMI-1640 (Life Technologies Inc.) supplemented with 10% FBS and 100 μM Homocysteine (Sigma, St. Louis, MO, USA).

2.2. Reagents

Antibody to ERK1/2 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody to JNK1 was purchased from BD PharMingen (San Diego, CA, USA). Antibody to phospho-ERK1/2 was purchased from Cell Signaling Technology (Beverly, MA, USA).

2.3. Plasmids

JNK1 and JNK1-APF expression vectors [40] were gifts from Dr Roger Davis, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School and Howard Hughes Medical Institute. Both JNK1 and JNK1-APF are driven by the CMV promoter. JNK1-APF contains mutations within two phosphorylation sites: threonine 183 replaced by alanine and tyrosine 185 replaced by phenylalanine. Bcl-XL and Bcl-2 expression vectors were gifts from Dr Tse-Hua Tan, Department of

Microbiology and Immunology, Baylor College of Medicine, Houston.

2.4. Western blot analysis

Aliquots of samples with 50 μg of protein, determined by the Bradford assay (BioRad, Hercules, CA, USA), were mixed with loading buffer (final concentrations of 62.5 mM Tris–HCl (pH 6.8), 2.3% sodium dodecyl sulphate (SDS), 100 mM dithiothreitol, and 0.005% bromophenol blue), boiled, fractionated in a 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a 0.45-μm nitrocellulose membrane by electroblotting (BioRad). The membranes were blocked with 2% fat-free milk in phosphate buffered saline (PBS), and then probed with primary antibody (0.05 μg/ml IgG) in PBS containing 0.1% Tween 20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horseradish peroxidase-conjugated $F(ab')_2$ of secondary antibody (BioRad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL Western blotting detection system (Amersham Co., Arlington Height, IL, USA).

2.5. Immune complex JNK1 kinase assay

Immunoprecipitation followed by JNK1 kinase assay was performed according to the manufacturer's protocol (Santa Cruz Biotechnology Inc.). Briefly, 0.5 mg of cell lysate was incubated with 0.5 μg of anti-JNK1 antibody (Santa Cruz Biotechnology Inc.) for 1 h in 1 ml RIPA buffer (PBS; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; 0.1 mg/ml phenylmethane sulfonyl fluoride (PMSF); 30 μl/ml aprotinin; 1 mM sodium orthovanadate). Subsequently, 20 μl protein A-Sepharose 4B conjugate (Zymed Laboratories Inc. South San Francisco, CA, USA) was added and the mix was incubated at 4°C on a rocking platform overnight. After washing the immune complexes four times in RIPA buffer, the beads were resuspended in 23 μl of kinase buffer (50 mM Tris–HCl, pH 7.5; 10 mM $MgCl_2$; 1 mM dithiothreitol (DTT)). One microgram of c-Jun, 1 μM of adenosine triphosphate (ATP), and 20 μCi of [γ-^{32}P]-ATP (7000 Ci/mmol) were added to a final volume of 25 μl. The reaction was incubated at 30°C for 30 min and stopped by addition of SDS sample buffer. After boiling, samples were fractionated through a 12% SDS-polyacrylamide gel. The gel was then directly exposed to X-ray film.

2.6. Transient transfection cell death assay

This assay was performed as described previously [38]. PC-3 cells were seeded in six-well tissue culture plates. The next day, cells were transiently cotransfected with a vector expressing LacZ gene plus an expression vector containing the genes of interest by lipofectin-mediated transfection according to a protocol by Life Technologies Inc. After transfection, the cells were cultured in the complete medium or methionine-free medium for 6 days. Fresh medium was placed on the cells every 3 days. Cells expressing lacZ were visualized by β-gal staining according to the manufacturer's protocol (Invitrogen Corporation, San Diego, CA, USA). Briefly, cells were washed once with PBS and fixed for 10 min in buffer containing 2% formaldehyde and 0.2% glutaraldehyde in PBS. After washing twice with PBS, the cells were incubated with an X-gal staining solution (4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM magnesium chloride, 1 mg/ml of X-gal in PBS) at 37°C until cells turn blue (approximately 3 h). Blue cells per 10× field, which reflected the total number of surviving cells, were counted and averaged from three random fields. Each experiment was repeated at least three times.

3. Results

3.1. Activation of JNK1 in response to methionine restriction in PC-3 human prostate cancer cells

JNK1 kinase activity increased in PC-3 cells within 6 days of methionine restriction and continued to increase for up to 9 days, as determined by an in vitro kinase assay (Fig. 1A). JNK1 kinase activity also increased in Hela cervical carcinoma cells, another methionine-dependent cell line (Fig. 1A), suggesting that JNK1 is a common mediator of cell death in response to methionine restriction in multiple types of cancer. JNK1 protein levels were not significantly changed in response to methionine restriction (Fig. 1B), suggesting that increased kinase activity was due to posttranslational modification. To deter-

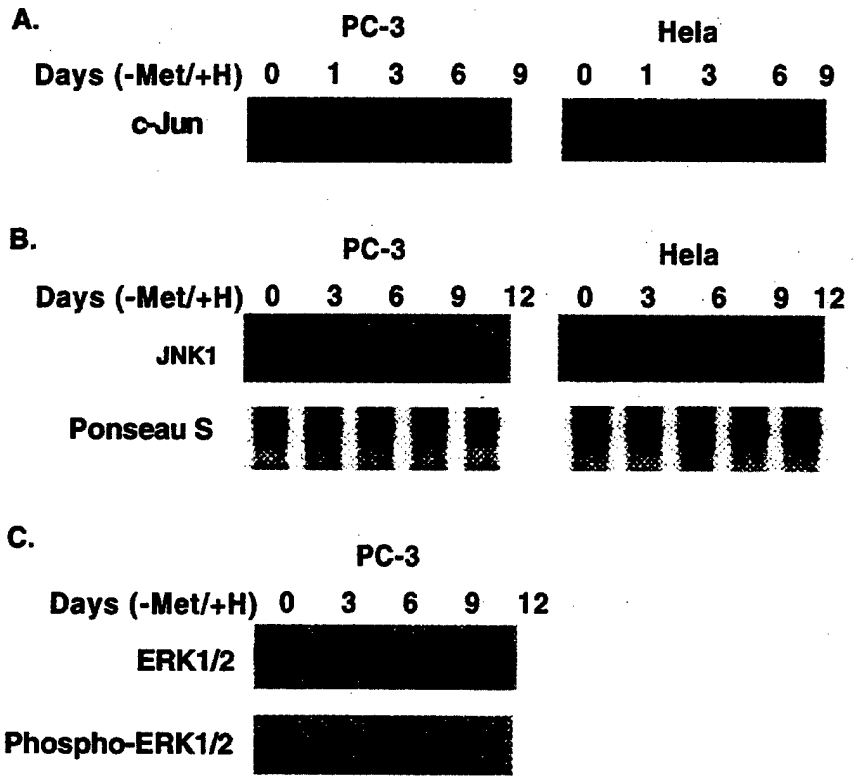

Fig. 1. Enhancement of JNK1 kinase activity by methionine restriction. (A) JNK1 kinase activity was determined by immune complex protein kinase assay as described in Section 2. Cell extracts used for the in vitro kinase assay were prepared from PC-3 and Hela cells cultured in complete or methionine-restricted medium up to 9 days. (B) Determination of JNK1 protein levels by Western blot analysis in PC-3 and Hela cells in response to methionine restriction. Ponseau S staining was used as protein loading control. (C) Determination of ERK1 kinase activity following methionine restriction for up to 12 days. The upper panel shows protein levels of ERK1and ERK2 in PC-3 cells. The lower panel shows phosphorylated ERK1/2 in response to methionine restriction by Western blot analysis using an anti-phospho-ERK1/2 antibody. For each experiment, fresh methionine-free medium was placed on cells every 3 days.

mine the specificity of JNK1 activation by methionine restriction, we measured activation of ERK1 and ERK2, other MAP kinases not usually activated by stress factors [41]. Phosphorylation of ERK1 and ERK2 at Thr202 and Tyr204, which directly correlates with their kinase activities [42], was not significantly altered by methionine restriction (Fig. 1C).

3.2. Essential role of the JNK1 signal transduction cascade in induction of cancer cell apoptosis by methionine restriction

Using an established transient transfection cell death assay [38], we found that over expression of JNK1 enhanced PC-3 cell apoptosis in response to methionine deprivation, whereas it had no effect on PC-3 cell viability under normal cell culture conditions (Fig. 2A). Methionine restriction alone reduced cell viability by approximately 70% (Fig. 2A). We next found that a kinase inactive mutant of JNK1, JNK1-APF, protected PC-3 cells from the proapoptotic effects of methionine restriction (Fig. 2A). JNK1-APF contains mutations within two phosphorylation sites: threonine 183 replaced by alanine and tyrosine 185 replaced by phenylalanine [40]. The protective effect of JNK1-APF was inhibited in a dose-dependent manner by transfection of wild type JNK1, suggesting that JNK1 and JNK1-APF compete with each another (Fig. 2B). Transfection of either Bcl-2 or Bcl-XL, two molecules known to inhibit apoptosis by blocking cytochrome c release from mitochondria and activation of caspases [43], inhibited cell death in

response to methionine restriction plus JNK1 over expression (Fig. 3). These data suggest that methionine restriction activates a mitochondria-dependent cell death pathway.

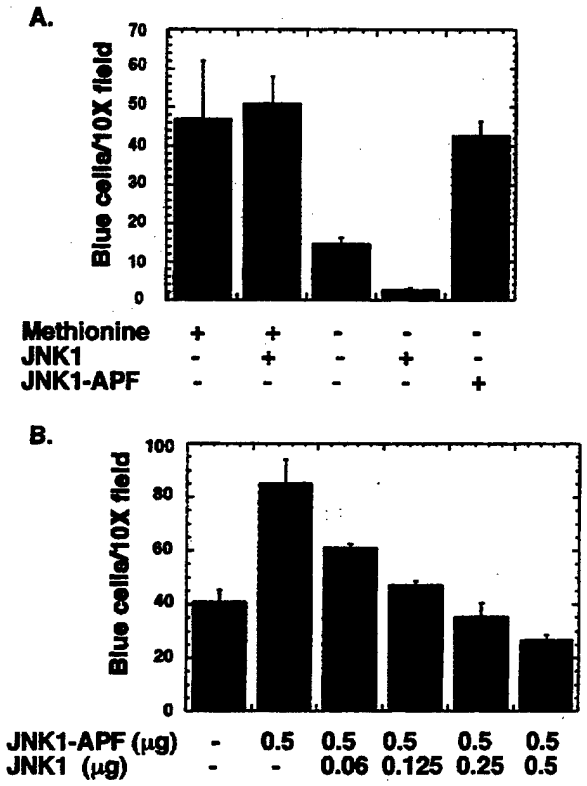

Fig. 2. Induction of apoptosis by JNK1 in PC-3 cells in response to methionine restriction. JNK1-induced apoptosis was determined by transient transfection cell death assay, as described in Section 2. (A) PC-3 cells were cotransfected with 0.75 μg reporter construct pCMV-lacZ plus either 0.75 μg of pCMV-JNK1 vector, 0.75 μg of pCMV-JNK1-APF vector, or 0.75 μg empty pCMV vector by lipofectin-mediated transfection. In each case, a total of 1.5 μg plasmid DNA were transfected. Subsequently, the cells were cultured in the presence or absence of methionine for 6 days. Fresh methionine-free or control medium was placed on cells every 3 days. By the end of cell culture, the cells expressing lacZ gene were visualized by β-gal staining. Blue cells were counted under the microscope. (B) PC-3 cells were cotransfected with 0.5 μg of pCMV-lacZ reporter construct plus 0.5 μg of pCMV-JNK1-APF and increasing quantities of pCMV-JNK1, as indicated. In each case, a total of 1.5 μg DNA were transfected, with empty pCMV vector accounting for the balance of DNA. After transfection, the cells were cultured in the absence of methionine for 6 days, followed by β-gal staining and cell counting. Fresh methionine-free medium was placed on cells every 3 days during the 6-day period. Bars represent standard deviation of at least three separate experiments.

4. Discussion

The current studies elucidate the apoptotic signaling pathway activated in human prostate cancer cells by methionine restriction. We found that JNK1kinase activity increased in PC-3 cells in response to methionine restriction. Over expression of JNK1 enhanced cell death, whereas a kinase inactive mutant of JNK1 blocked cell death induced by methionine restriction. The consequence of JNK1 signaling is cell context-dependent, with growth, differentiation, or apoptosis as potential outcomes. Microtubule-damaging drugs, such as taxol, activate JNK1, resulting in apoptosis of multiple types of cancer cells [44,45]. Serum or nerve growth factor deprivation activates JNK1 activity and induces apoptosis in PC12 cells [39]. Anticancer drugs, UV, and γ-radiation also induce the stress response and apoptosis [45,46].

We found that over expression of Bcl-2 and Bcl-XL inhibited PC-3 cell apoptosis induced by methionine

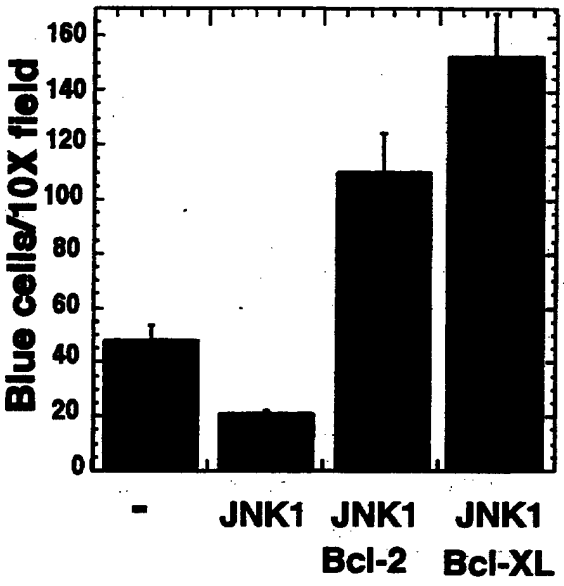

Fig. 3. Bcl-2 and Bcl-XL block JNK1-induced apoptosis in response to methionine restriction in PC-3 cells. PC-3 cells were cotransfected with 0.5 μg of pCMV-lacZ construct ±0.5 μg of pCMV-JNK1 ±either 0.5 μg pCMV-Bcl-2 or pCMV-Bcl-XL. In each case, a total of 1.5 μg DNA were transfected, with the balance coming from pCMV empty vector. After transfection, cells were cultured in the methionine-free medium for 6 days, followed by β-gal staining and cell counting. Fresh methionine-free medium was placed on cells every 3 days. Bars represent standard deviation of at least three separate experiments.

restriction. Two major functions have been ascribed to Bcl-2 and Bcl-XL [43,47]. First, they dimerize with Bax and thereby inhibit its proapoptotic function. Second, they interact with Apaf-1 to inhibit binding and activation of caspase 9 [48]. Consequently, Bcl-2 and Bcl-XL inhibit cytochrome c release from mitochondria and block activation of the mitochondria-dependent caspase cascade [31]. Our results suggest that cell death in response to methionine restriction is mitochondria-dependent. However, additional studies will be required to confirm that possibility.

Cancer cell death in response to methionine restriction occurs gradually over several days and seems to involve at least three overlapping stages (Fig. 4). Cells first undergo cell cycle arrest primarily in G2/M [49], then activate JNK1, and finally undergo programmed cell death, as evidenced by cleavage of DNA into 'nucleosomal ladders' [15,49]. The current studies suggest that JNK1 activation during the second stage signals prostate cancer cells to commit to death, after which they undergo active disassembly. The fact that each of the three stages occurs gradually over several days suggests that the time at which a cancer cell enters each stage depends upon its position in the cell cycle when methionine is restricted. This possibility will need to be tested in future experiments.

Mechanistic studies concerning methionine dependence of tumors are certainly clinically relevant in the near term, since many preclinical studies indicate that dietary and enzymatic methionine restriction has anti-

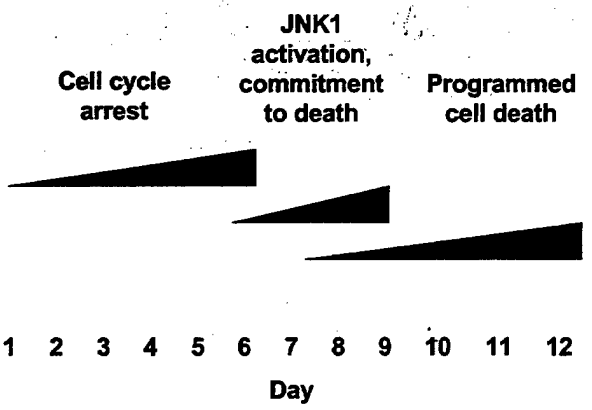

Fig. 4. Schematic representation of the sequence of events leading to prostate cancer cell apoptosis in culture following methionine restriction.

tumor activity in tumor bearing animals. In fact, a phase I clinical trial of dietary methionine restriction is currently underway at our institution, and preliminary results appear promising. However, methionine dependence of tumors represents more than just an opportunity to 'starve' tumors. Rather, methionine dependence is an example of a hallmark feature of cancer, namely abnormal nutrient metabolism of tumors. Recent mechanistic studies concerning other hallmark features of cancer, such as cell cycle abnormalities and tumor angiogenesis, led to development of drugs that target extracellular growth factor receptors and tumor vessels. Hopefully studies concerning the fundamental mechanisms underlying methionine dependence of tumors will also result in the discovery of novel therapeutic targets.

Acknowledgements

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Induction of caspase-dependent and -independent apoptosis in response to methionine restriction

SHAN LU, SARA M. HOESTJE, EUGENE CHOO and DANIEL E. EPNER

Department of Medicine, Baylor College of Medicine and VA Medical Center, Houston, TX 77030, USA

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Abstract. Tumor cells are more sensitive to methionine restriction than normal tissues, a phenomenon known as methionine auxotrophy. Previous studies have demonstrated that methionine restriction causes tumor cell growth arrest and eventually apoptosis. The current studies were undertaken to elucidate the molecular pathways leading to apoptosis induced by methionine restriction. We found that methionine restriction induced formation of oligonucleosomal DNA fragment and cytochrome c release from mitochondria in methionine-dependent PC3 and Hela cells. Methionine restriction also led to cleavage and activation of initiator and effector caspases in Hela cells but not PC3 cells. Furthermore, methionine restriction resulted in cleavage of BID and reduction in Bcl-2 levels in both cell lines. These data suggest that apoptosis induced by methionine restriction is mitochondria-dependent. Methionine restriction induced caspase-independent cell death in PC3 cells, whereas it stimulated caspase-dependent cell death in Hela cells. Cleavage of BID and decreased expression of Bcl-2 upon methionine deprivation may be the underlying mechanism to stimulate release of cytochrome c from mitochondria.

Introduction

Methionine dependence or methionine auxotrophy is a consequence of oncogenic transformation and a metabolic defect in cancer cells (1,2). This defect has been found in many, if not in all, types of human tumors. Methionine is an essential amino acid that functions as a building block for proteins, a precursor of glutathione, an aminopropyl donor for synthesis of polyamines, and the major methyl donor for methylation of DNA, protein, and other molecules. Glutathione is a tripeptide that protects cells from oxidative stress, whereas polyamines have far-ranging effects on nuclear structure and cell division. Normal mammalian cells and methionine-independent tumor cells proliferate normally in culture medium with low level of methionine supplemented with homocysteine, a nonstandard amino acid with the same structure as methionine except lacking a methyl group (3). Animals fed diets in which methionine has been replaced by homocysteine suffer no ill effects and grow normally (4,5). In contrast, the majority of human and rodent tumors are dependent on methionine, since they fail to grow in culture medium with low level of methionine supplemented with homocysteine (6-9). Dietary methionine restriction causes regression of various tumor xenografts in nude mice (10,11), and inhibits metastasis in animal models (4,10). Methioninase, an enzyme that degrades methionine and homocysteine, also inhibits growth of solid tumors in animals and synergizes with chemotherapy drugs (12-16). A recent clinical trial showed that dietary methionine restriction is safe and feasible in adults with metastatic cancer, and results in significant reduction of plasma methionine level (17).

The molecular mechanisms underlying methionine dependence of cancer cells have not been elucidated. Both normal and cancer cells can convert homocysteine to methionine catalyzed by either 5-methyltetrahydrofolate-homocysteine methyltransferase or betaine-homocysteine methyltransferase (1,2). The methyltransferase activities of methionine-dependent tumor cells are comparable with or higher than normal cells and methionine-dependent tumor cells (1,18). Methionine dependence may be due to elevated rates of transmethylation in cancer cells (2,19).

Methionine restriction has a cytostatic effect on cancer cells at early stage, causing the tumor cells to arrest predominantly in the G2/M phase of the cell cycle (11,20,21). Eventually, methionine restriction induces apoptosis (21). Previous studies demonstrated that methionine restriction inhibits brain tumor growth at least partly by downregulating the DNA repair gene O^6-methylguanine-DNA methyltransferase (MGMT or also named O^6-alkylguanine-DNA alkyltransferase) (22,23). In addition, methionine restriction sensitizes tumor cells to chemotherapy drugs and inhibits drug resistance by decreasing ATP pool and GSH content (24).

Apoptosis occurs through either mitochondria-dependent or -independent pathway (25). Mitochondria-dependent apoptosis is generally initiated by various environmental and genotoxic stress factors, such as ultraviolet (UV) light, γ-irradiation, hydrogen peroxide, microtubule-damaging drugs, protein synthesis inhibitors, and DNA-damaging drugs

Correspondence to: Dr Daniel E. Epner, VA Medical Center, Medical Service (111H), 2002 Holcombe Blvd., Houston, TX 77030, USA
E-mail: depner@bcm.tmc.edu

Key words: methionine restriction, apoptosis, caspase

(26-29). Recent studies have demonstrated that these factors activate the mitogen-activated protein kinase (MAPK) kinase pathway or stress response kinase pathway, resulting in the mitochondria-dependent apoptosis (30-32). Activation of JNK1, a key stress response kinase, induces mitochondria-dependent apoptosis by cleavage of BID through unknown signaling pathways, resulting in cytochrome c release from the mitochondria into the cytosol (33,34). Cytochrome c release is required for cleavage of caspase 9 proenzyme to its active form, which initiates the caspase cascade by cleaving and activating downstream (effector) caspases, such as 3 and 6 (35). Mitochondria-independent apoptosis is initiated by cell membrane death receptors upon ligand binding, such as binding of the Fas ligand to its receptor (36). This event causes protein assembly at the intracellular domain of the receptor, cleavage of the caspase 8 proenzyme to its active form, and initiation of the caspase cascade. Crosstalk exists between the mitochondria-dependent and -independent apoptotic pathways. For example, caspase 8 cleaves and activates BID, a proapoptotic member of the Bcl-2 family of proteins, which triggers cytochrome c release from mitochondria (37,38). Regardless of whether apoptosis occurs through mitochondria-dependent or -independent pathways, effector caspases dismantle cells by cleaving proteins that normally function to maintain cell structure and genomic integrity. For instance, caspase 3 cleaves the substrate ICAD, a protein that complexes with and inhibits the caspase-activated deoxyribonuclease (CAD, or DNA fragmentation factor, DFF) (39-41). Upon ICAD degradation, CAD is released from the complex and translocated to the nucleus, where it cleaves genomic DNA, resulting in the hallmark oligonucleosomal DNA.

Previously, we demonstrated that activation of the cellular stress response by methionine restriction is responsible for apoptosis in prostate cancer cells (42). The current studies were undertaken to identify the apoptotic pathways induced by methionine restriction in cancer cells. We found that methionine restriction activated caspase-dependent cell death in Hela cervical carcinoma cells, whereas it activated caspase-independent cell death in PC3 prostate carcinoma cells.

Materials and methods

Cell culture. Human cervical cancer Hela cells and human prostatic cancer PC3 cells (American Type Culture Collection) were maintained in RPMI-1640 (Life Technologies, Inc.) supplemented with 10% FBS (HyClone Laboratories, Logan, UT) at 37˚C in 5% CO_2. Methionine restriction experiments were performed in methionine-free RPMI-1640 (Life Technologies, Inc.) supplemented with 10% FBS and 100 µM Homocysteine (Sigma).

Reagents. Antibodies for caspases 3, 6, 8, and 9, Bcl-2, and BID were purchased from Santa Cruz Biotechnology, Inc. Antibody for cytochrome c was obtained from BD PharMingen.

Western blot analysis. Aliquots of samples with 50 µg of protein, determined by the Bradford assay (BioRad), were mixed with loading buffer [final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 100 mM dithiothreitol, and 0.005% bromophenol blue], boiled, fractionated in a 15% SDS-PAGE, and transferred onto a 0.45-µm nitrocellulose membrane by electroblotting (BioRad). The membranes were blocked with 2% fat-free milk in PBS, and then probed with first antibody (0.05 µg/ml IgG) in PBS containing 0.1% Tween 20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horseradish peroxidase-conjugated F(ab')2 of secondary antibody (BioRad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL Western blotting detection system (Amersham Co.).

Hypotonic lysis for cytochrome c Western blot analysis. Determination of cytochrome c release from mitochondria upon activation of apoptosis was performed as described (43). Cells were trypsinized, pelleted at 1500 rpm for 5 min, washed twice with PBS, lysed in 300 µl hypotonic buffer [220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, protease inhibitors], and incubated on ice for 45 min. The lysates were homogenized by trituration 20 times and centrifuged at 14000 rpm for 10 min. Supernatants were collected and subjected to protein quantitation, followed by Western blot analysis using an anti-cytochrome c antibody.

DNA fragmentation assay. Genomic DNA was isolated using a kit purchased from Qiagen (Chatsworth, CA). Ten micrograms of DNA per lane were electrophoresed through 1.8% agarose gel and the gel was stained with ethidium bromide. Fluorescent DNA bands were visualized with an ultraviolet transilluminator and photographed.

Caspase assay. Caspase assays were performed according to the manufacturer's protocol (BioRad). Briefly, the assays were based on the ability of activated caspase to cleave a fluorogenic peptide containing caspase cleavage site, consequently releasing the fluorogenic molecule. Fluorogenic tetrapeptide Ac-LEHD-AFC is substrate for caspases 4, 5 and 9, whereas fluorogenic tetrapeptide Ac-DEVD-AFC is substrate for caspases 3, 6, 7, 8, and 10 (Calbiochem-Novabiochem Corporation).

Results

Activation of caspase-dependent and -independent apoptosis in cell type specific manner upon methionine restriction. Previous studies established that methionine restriction induced apoptosis in PC3 cells, culminating in a typical nucleosomal DNA ladder (21). In the current studies, we found that methionine restriction also induced oligonucleosomal DNA formation in Hela cells (Fig. 1). We also found that cytosolic levels of cytochrome c increased after 3 days of methionine restriction in both PC3 and Hela cells (Fig. 2). By 9 days, cytochrome c levels peaked. These data suggest that apoptosis in response to methionine restriction is mitochondria-dependent.

We next examined whether methionine restriction caused activation of caspases, including initiator caspases 8 and 9

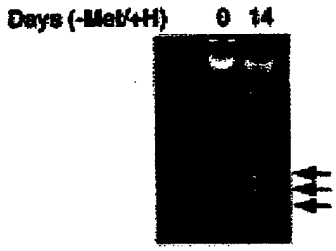
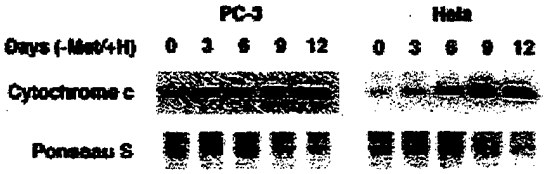


Figure. 1. Methionine restriction induces apoptosis in Hela cells. Hela cells were grown for 14 days in RPMI medium containing 100 μM methionine or in methionine-free RPMI medium containing 100 μM homocysteine. Both media contain 10% fetal calf serum. Genomic DNA was isolated and analyzed by gel electrophoresis. Arrows indicate the fragmented DNA. Leftmost lane is DNA molecular weight standards.



FIigure. 2. Methionine restriction induces cytochrome c release from mitochondria in PC-3 and Hela cells. Determination of cytochrome c release upon activation of apoptosis was performed by hypotonic lysis for cytochrome c Western blots. The cells grown in the presence or absence of methionine for the indicated times were harvested for preparation of cytosolic protein extracts, followed by Western blot analysis using an anti-cytochrome c antibody. Ponseau S staining was used as protein loading control.

and effector caspases 3 and 6. We found that methionine restriction had little if any effect on levels of these caspases in PC-3 cells (Fig. 3A, left panel). In contrast, caspase 3, 6, and 9 proenzyme levels decreased upon methionine restriction in a time-dependent fashion in Hela cells (Fig. 3A, right panel). In contrast, caspase 8 proenzyme levels were unaffected in both cell lines. Despite the fact that caspase 3, 6, and 9 proenzyme levels fell in Hela cells in response to methionine restriction, the active forms of all three caspases were undetectable by Western blot analysis. Inability to detect cleaved, active caspases was not due to technical problems with the Western blot procedure, since the active form of caspase 9 was detectable following treatment of Hela cells with staurosporine, a compound known to rapidly induce apoptosis (Fig. 3B). Both proenzyme and active forms of caspase 9 were detected within four hours of staurosporine treatment (Fig. 3B). These results suggest that active forms of caspases were not detectable in methionine restricted cells as a result of their lability and rapid degradation relative to the very gradual pace of cell death.

Consistent with the Western blot analyses, caspase activities were increased in Hela cells in response to methionine restriction (Fig. 4). Cleavage of peptide Ac-LEHD-AFC, which is a substrate for caspases 4, 5, and 9, increased transiently after six days of methionine restriction (Fig. 4A). The observed increase in caspase activity after six days of methionine restriction was probably a reflection of caspase 9

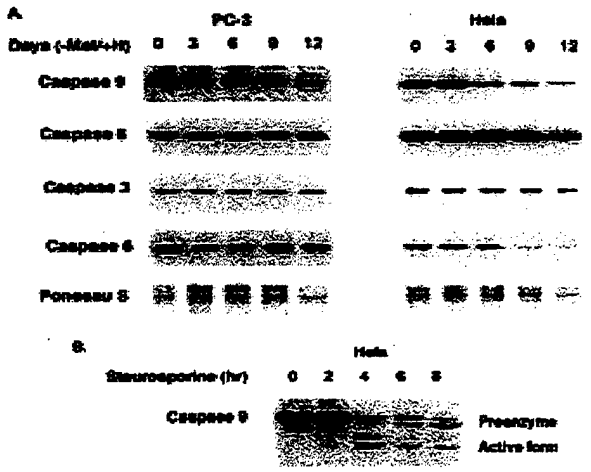


Figure. 3. Cleavage of caspase proenzymes in response to methionine restriction in PC-3 and Hela cells. (A), Determination of caspase proenzyme level. Cell extracts were prepared from PC-3 or Hela cells cultured in the presence or absence of methionine for 3, 6, 9, and 12 days. Proenzyme levels of caspases 3, 6, 9, and 8 were determined by Western blot analysis using corresponding antibodies. Ponseau S staining was used as protein loading control. (B), Caspase 9 cleavage in response to staurosporine. As a control, proenzyme and active form of caspase 9 were also determined in Hela cells treated with staurosporine (2 μM) for 2, 4, 6, and 8 h by Western blot analysis.

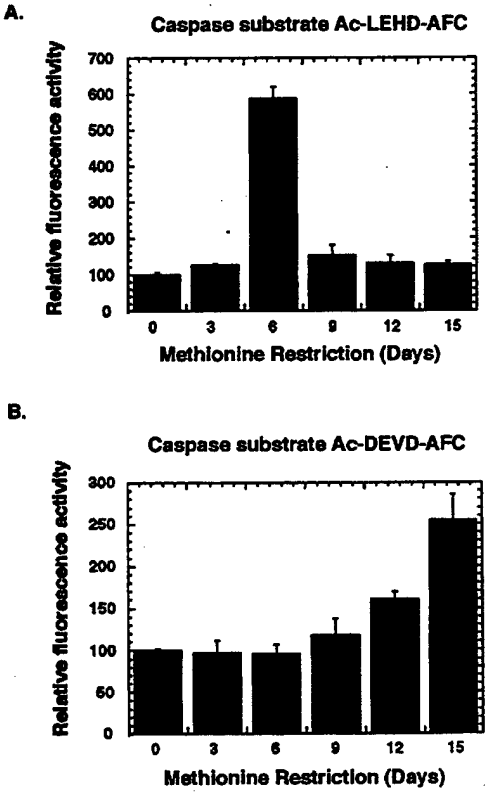


Figure. 4. Determination of caspase activities in Hela cells. Cell extracts from control and methionine-restricted cells for 3, 6, 9, 12, and 15 days were used for caspase assays as described in the Materials and methods. Increased relative fluorescence activity as compared with control sample was the indicator of caspase activity. The results are expressed as caspase activity, which is a mean of duplicate samples ± standard error. Each experiment was repeated at least 5 times.

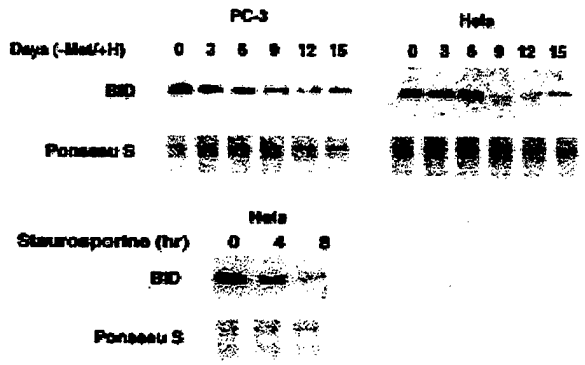


Figure. 5. Expression of BID in response to methionine restriction in PC-3 and Hela cells. Cleavage of BID was detected upon methionine restriction by Western blot analysis in both PC-3 and Hela cells (upper panel). The lower panel is a control showing that treatment of staurosporine (1 μM) for 4 and 8 h induces BID cleavage in Hela cells.

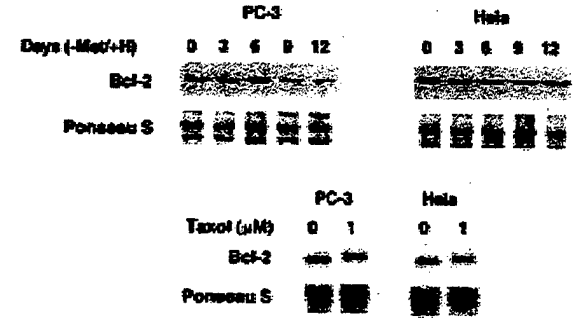


Figure. 6. Expression of Bcl-2 in response to methionine restriction in PC-3 and Hela cells. Bcl-2 expression was determined by Western blot analysis upon methionine restriction in PC-3 and Hela cells (upper panel). As a control, Taxol treatment (1 μM) for 24 h induced phosphorylation of Bcl-2 in both cell lines as demonstrated by a supershifted band (lower panel). Ponseau S staining was used as protein loading control.

activity, since caspase 9 proenzyme was degraded at the same time point (Fig. 3A). In addition, caspases 4 and 5 were undetectable by Western blot analysis even at baseline (data not shown). Cleavage of peptide Ac-DEVD-AFC, which is a substrate for caspases 3, 6, 7, 8, and 10, increased in a time-dependent manner in Hela cells in response to methionine restriction for up to 15 days (Fig. 4B). This increase probably reflected caspase 6 activity, since caspase 6 proenzyme was almost entirely degraded in Hela cells with similar kinetics. However, caspases 3, 7, 8, and 10 may have also contributed to varying degrees. Caspase activity was not detected in PC-3 cells in response to methionine restriction (data not shown). These data suggest that Hela cells undergo caspase-dependent cell death, whereas PC-3 cells undergo caspase-independent cell death in response to methionine restriction.

Bcl-2 and BID are involved in the apoptosis induced by methionine restriction. To determine the underlying mechanisms of crosstalk between the stress response and mitochondria-dependent cell death pathway, we measured levels of BID and Bcl-2 in response to methionine restriction. BID activation following cleavage is known to induce apoptosis by acting on mitochondria, thereby releasing cytochrome c. BID has also been shown to be activated as a consequence of JNK1 activation (33). We found that methionine restriction induced BID cleavage in a time-dependent manner in both PC-3 and Hela cells (Fig. 5, upper panel). Staurosporine-treated Hela cells were used as a positive control for BID cleavage (Fig. 5, lower panel) (44). In addition, Methionine restriction resulted in a slight decrease in Bcl-2 protein levels in PC-3 and Hela cells (Fig. 6, upper panel). However, Bcl-2 did not become phosphorylated (inactivated) in response to methionine restriction. Taxol-treated PC-3 and Hela cells were used as positive controls for Bcl-2 phosphorylation (45,46), as indicated by upward shift of Bcl-2 band on Western blot (Fig. 6, lower panel). These data suggest that activation of BID and decreased Bcl-2 abundance led to initiation of the mitochondria-dependent cell death pathway in response to methionine restriction.

Discussion

The current studies elucidate the apoptotic signaling pathway activated in PC3 and Hela cells by methionine restriction. We found that methionine restriction caused cytochrome c release from mitochondria in PC-3 and Hela cells, suggesting that cell death in response to methionine restriction is mitochondria-dependent. Bcl-2 and Bcl-XL, antiapoptotic molecules of Bcl-2 family proteins, are known to block cytochrome c release from mitochondria (47,48). They do so by dimerizing with Bax to inhibit its proapoptotic action and interacting with Apaf-1 to inhibit binding and activation of caspase 9 (49). Consequently, Bcl-2 and Bcl-XL inhibit cytochrome c release from mitochondria and activation of the mitochondria-dependent cell death (26). We previously demonstrated that overexpression of Bcl-2 and Bcl-XL inhibited PC-3 cell apoptosis induced by methionine restriction, suggesting that death in that context is mitochondria-dependent (42).

In the current studies, caspase 8 proenzyme levels were unaffected by methionine restriction in both PC-3 and Hela cells, suggesting that activation of the caspase cascade by methionine restriction is not membrane death receptor(Fas)-dependent. In PC-3 cells, cleavage and enzymatic activities of initiator caspases 8 and 9 and effector caspases 3 and 6 were essentially unaffected by methionine restriction. In contrast, caspases 3, 6, and 9 were cleaved and/or enzymatically activated in Hela cells in response to methionine restriction. These data suggest that PC-3 and Hela cells undergo two different forms of cell death in response to methionine restriction, both of which are mitochondria-dependent. PC-3 cells undergo caspase-independent programmed cell death, whereas Hela cells undergo caspase-dependent programmed cell death. Both death pathways ultimately result in DNA fragmentation (42).

Caspase-independent programmed cell death is a recently described mode of cell death that morphologically resembles necrosis, but that is distinguishable biochemically from both classical apoptosis (i.e. caspase-dependent programmed cell death) and necrosis (i.e. unprogrammed cell death) (50).

A few factors, such as TAJ (toxicity and JNK1 inducer), oncogenic Ras, and oxidative stress (51-53), are known to induce caspase-independent programmed cell death. Staurosporine is known to induce both caspase-dependent and -independent apoptosis (54), as methionine restriction does. DNA fragmentation is seen in both caspase-independent and caspase-dependent cell death (55). Proapoptotic factors still induce DNA fragmentation in cells from transgenic mice lacking CAD (caspase-activated deoxyribonuclease or DNA fragmentation factor), suggesting that one or more deoxyribonucleases in addition to CAD exist (56,57). In supporting this possibility, Wang's group recently identified endonuclease G, a mitochondria-specific nuclease that translocates to the nucleus to cleave chromatin into nucleosomal fragments during apoptosis (58). This activity is independent of caspases and represents at least one mechanism underlying caspase-independent apoptosis.

Methionine restriction activates the stress response and eventually induces apoptosis in methionine-dependent cancer cells (42). The molecular mechanisms of crosstalk between the stress response and apoptotic pathways have not been well elucidated. Korsmeyer's group demonstrated that microtubule-damaging drugs activate JNK1 to phosphorylate and inactivate Bcl-2 at the G2/M phase of the cell cycle, which may make cells susceptible to death (59). The phosphorylation sites in Bcl-2 were identified at Ser70, Ser87 and Thr69. Therefore, phosphorylation of Bcl-2 by JNK1 inhibits its antiapoptotic activities and consequently activates the mitochondria-dependent apoptotic pathway. Davis's group showed that JNK1 and JNK2 deficient cells failed to activate mitochondria-dependent cell death pathway by cleavage of BID and release of cytochrome c from the mitochondria in response to ultraviolet radiation (33). That study suggested that induction of BID cleavage by JNK1 is required for cell death. Previous studies from our lab indicate that cell death in response to methionine restriction is similarly dependent upon JNK1 activation (42). These studies combined with the current ones showing BID cleavage suggest that BID is the mediator between the stress response pathways and mitochondria-dependent cell death pathway (42).

Acknowledgements

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Glutathione S-Transferase pi Is Upregulated in the Stromal Compartment of Hormone Independent Prostate Cancer

Ming Li,[1,4,5] Michael M. Ittmann,[2,4,5] David R. Rowley,[3,4] Anne A. Knowlton,[1,4,5] Ajula K. Vaid,[2,4] and Daniel E. Epner[1,4]*

[1]Department of Medicine, Houston, Texas
[2]Department of Pathology, Houston, Texas
[3]Department of Molecular and Cellular Biology, Houston, Texas
[4]Baylor College of Medicine and [5]VA Medical Center, Houston, Texas

BACKGROUND. Glutathione *S*-transferase (GST) pi is a detoxifying enzyme abundant in normal prostate basal cells but only rarely expressed in prostate cancer cells. The current studies are the first to focus on GST pi in the stromal compartment of prostate tumors.
METHODS. We employed immunohistochemical, immunofluorescence, and Western blot analysis to measure GST pi expression and subcellular localization in 21 primary and metastatic tumors from patients with hormone independent prostate cancer, as well as seven lymph node metastases and six prostatectomy specimens.
RESULTS. GST pi was detectable in stromal cells in 17 of the 21 hormone independent prostate tumors. GST pi tissue distribution in hormone independent tumors coincided with vimentin staining, suggesting that GST pi is expressed by reactive fibroblasts and/or myofibroblasts.
CONCLUSIONS. The current results suggest that prostate cancer cells induce an injury response in the stroma during progression to hormone independence, which results in GST pi expression. Stromal GST pi may contribute to chemoresistence of advanced prostate cancer.
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KEY WORDS: **glutathione transferase; prostatic neoplasms; stromal cells**

INTRODUCTION

Glutathione S-transferases (GSTs) are a multi-gene family of enzymes involved in the detoxification and activation of a wide variety of chemicals, including oxygen free radicals and peroxides produced by physiological cellular processes [1]. All eukaryotic species possess multiple cytosolic and membrane-bound GST isoenzymes. The cytosolic enzymes are encoded by at least five distantly related gene families, designated alpha, mu, pi, sigma, and theta. The most abundant mammalian GSTs are alpha, mu, and pi. GSTs catalyze the general reaction: GSH + R-X → GSR + HX, where GSH is glutathione and RX is an electrophilic substrate [2]. In so doing, they bring GSH and the electrophilic substrate into close proximity and activate the sulfhydryl group on GSH, thereby allowing for nucleophilic attack of GSH on the electrophilic substrate. The formation of a thioether bond between the cysteine residue of GSH and the electrophile usually results in a less reactive and more water soluble product. Thus, GSTs catalyze detoxification reactions.

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*Correspondence to: Daniel E. Epner, VAMC, Medical Service (111H), 2002 Holcombe Blvd., Houston, TX 77030.
E-mail: depner@bcm.tmc.edu

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GST expression is a crucial factor in determining sensitivity of normal cells to a broad spectrum of toxic chemicals. Individual isoenzymes contribute to resistance to environmental pollutants, carcinogens, and products of oxidative stress. Several studies have focused on the protective role of GST pi in preventing carcinogenesis [3]. Transient overexpression of GST pi inhibits the cytotoxic effects of cigarette smoke on lung cells [4]. GST pi also protects cells from death induced by reactive oxygen species by upregulating multiple stress kinases and suppressing proaptotic JNK signaling [5].

Besides having a protective role in normal tissues, GST pi is also the most abundant member of the GST family in human tumor cells [6]. Many human tumors and cancer cell lines contain increased GST pi levels relative to their tissues of origin [2,7,8], and GST pi expression in many tumors is inversely correlated with prognosis and response to chemotherapy [9]. Transfection of cells in culture with GST pi increases resistance to several chemotherapy drugs [10]. GST pi is therefore a "double-edged sword," since it not only protects normal tissues from carcinogens but also probably contributes to the multidrug-resistant phenotype observed in cancer [1,9–12].

In contrast to frequent overexpression of GST-pi observed in many types of cancer, the vast majority of primary human prostate tumors contain no detectable GST pi [13–16]. GST pi is abundant in normal prostate basal epithelial cells, but basal cells are lost during development of invasive cancer [13,14]. Absence of GST pi expression in human prostate cancer is accompanied by hypermethylation of regulatory sequences within the GST pi gene, whereas no such hypermethylation is present in normal tissues or benign prostatic hyperplasia [13,17,18]. Previous studies have focused on GST pi in radical prostatectomy specimens rather than on metastatic or hormone independent prostate cancer.

Despite lack of GST pi expression in most primary prostate tumors, four commonly studied hormone independent prostate cancer cell lines express high levels of GST pi as determined by Western blot analysis [13]. In contrast, hormone dependent LNCaP prostate cancer cells do not express GST pi. This finding suggests that GST pi may become re-expressed in hormone independent prostate cancer, which would be consistent with its known overexpression in many other tumors. We undertook the current studies to test that hypothesis. Using immunohistochemistry, we analyzed GST pi expression in several primary and metastatic prostate tumors from patients with hormone independent disease. We focused on GST pi expression in all tissue compartments, not just the epithelial compartment. Our results suggest that GST pi has a major role in prostate cancer progression despite the fact that its expression is lost in the early stages of prostate carcinogenesis.

MATERIALS AND METHODS

Patient Tissue Samples

The Baylor College of Medicine Institutional Review Board approved all studies. Thirty-four specimens were analyzed (Table I). Specimens were identified by number only in order to maintain patient confidentiality.

Immunohistochemistry

Six-micron sections of paraffin-embedded specimens were mounted on glass slides; deparaffinized by sequential treatment with xylene, absolute ethanol, 95% ethanol, and 80% ethanol; immersed in 0.01 M citrate buffer (pH 6.0) and placed in a steamer for 8 min for antigen retrieval; and rinsed three times with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4). Thereafter, all steps were done at room temperature in a humidification chamber. Slides were incubated with 1% hydrogen peroxide for 30 min to reduce background staining due to endogenous peroxidases; incubated in 3% normal goat serum in PBS for 1 hr to block nonspecific binding; incubated with polyclonal rabbit anti-human GST-pi antibody (Biogenesis, NH, USA, catalog number) at dilution of 1:100 in 3% normal goat serum in PBS for 3 hr; incubated with biotinylated goat anti-rabbit antibody at a dilution of 1:200 for 30 min; washed with PBS; incubated for 30 min with horseradish peroxidase-conjugated avidin-biotin complex according to manufacturer's directions (ABC-kit, Vector Laboratories, Burlingame, CA); and finally developed using 3-amino-9-ethylcarbazole, which yields a red color. Sections were then counterstained with hematoxylin, dehydrated, and mounted. The same procedure was used for analysis of stromal cell markers with mouse anti-human vimentin antibody (Chemicon International, Inc., Temecula, CA) diluted 1:50, mouse anti-human smooth muscle α-actin antibody (Sigma) diluted 1:200, or mouse anti-human smooth muscle calponin antibody (Sigma) diluted 1:2,000. Each of the antibodies was diluted in 3% normal goat serum in PBS.

Determination of Staining Intensity

Staining intensity was quantitated with SigmaScan Pro Image Analysis Software (SPSS, Chicago, IL). Red intensity, blue intensity, and light intensity values were determined for 100 randomly selected pixels from GST pi positive areas of two normal prostate specimens within basal cells and five hormone independent prostate cancer specimens within stromal

TABLE I. GST pi Expression, Distribution, and Intensity in Human Prostate Cancer Specimens

Specimen	Number	Only cancer cells+	Only stroma+	Subjective staining intensity of stroma[a]	Cancer cells and stroma+
Surgical specimens					
Prostatectomy	6	0	0	N/A	0
Lymph node	7	1	0	N/A	0
Total surgical specimens	13	1	0	N/A	0
Hormone independent					
Autopsy					
Prostate	5	0	3	1, 1, 2, 2, 3	2
Bone metastasis	3	1	1	3, 1	1
Lung metastasis	2	0	0	N/A	0
Liver metastasis	1	0	1	1	0
Adrenal metastasis	1	0	0	N/A	0
Surgical					
TURP[b]	9	0	9	1, 2, 2, 2, 3, 3, 3, 3, 3	0
Total hormone independent	21	1	14		3
Total specimens	34				

[a]Staining intensity ranged from 0 to 4, with 4 corresponding to normal basal cells, and 1 representing faint staining.
[b]Transurethral resection of prostate.
N/A, not applicable.
+indicates positive staining.

cells. The arbitrary intensity value was calculated by dividing mean uncorrected red value (signal) by mean uncorrected blue value (background/counterstain), and then dividing again by mean light transmittance. Light transmittance values ranged from 0 (black) to 255 (white). Relatively low light transmittance and high red/blue ratios correlated with greater GST pi abundance.

Immunofluorescence Microscopy

Six-micron sections of paraffin-embedded specimens were deparaffinized, treated with citrate buffer, and rinsed in PBS as described above. They were then incubated with 3% normal goat serum in PBS for 1 hr to reduce nonspecific binding. Primary antibody incubation was done for 3 hr with rabbit anti-human GST-pi antibody diluted 1:100 combined with mouse anti-human vimentin antibody (Chemicon International, Inc.) diluted 1:50. Primary antibodies were diluted in 3% normal goat serum in PBS. Slides were then washed three times with PBS and incubated with fluorescein-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR), diluted 1:200 in PBS and Texas Red goat anti-mouse antibody (Molecular Probes), diluted 1:200 in PBS for 1 hr, air-dried at room temperature, and covered with cover slips. Images were captured with a Spot CCD camera attached to an Olympus BX60 fluorescence microscope. Images were then modified with Adobe photoshop software (San Jose, CA) in order to reduce background and enhance contrast.

Western Blot Analysis

Aliquots of samples with 50 μg of protein, determined by the Bradford assay (BioRad, Hercules, CA), were mixed with loading buffer (final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 100 mM dithiothreitol, and 0.005% bromophenol blue), boiled, fractionated in a 15% SDS–PAGE, and transferred onto a 0.45-μm nitrocellulose membrane by electroblotting (BioRad). The membranes were blocked with 2% fat-free milk in PBS, and then probed with primary antibody (0.05 μg/ml IgG) in PBS containing 0.1% Tween 20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horseradish peroxidase-conjugated F(ab')2 of secondary antibody (BioRad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL Western blotting detection system (Amersham Co., Arlington Height, IL).

RESULTS

Immunohistochemical Detection of GST pi in Hormone Independent Human Prostate Cancer

Several previous studies established that GST pi is abundant in normal prostate basal cells but is absent from >90% of prostate tumors. We therefore used radical prostatectomy specimens as controls. As expected, we found that GST pi was abundant in the basal cell layer of normal glands (Fig. 1A) but was undetectable in cancer tissue of all six prostatectomy specimens

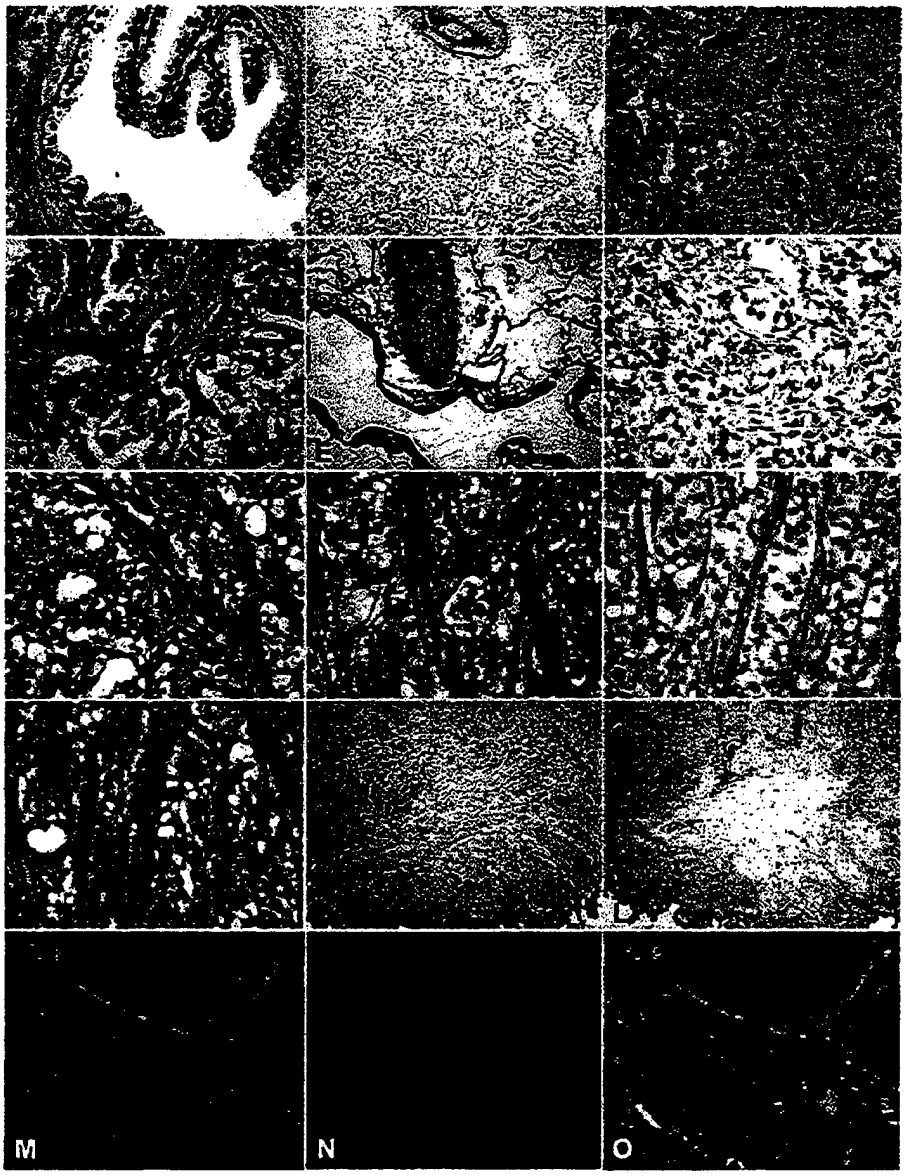

Fig. 1. Immunohistochemical (IHC) and immunofluorescence analysis of paraffin-embedded specimens for GST pi and stromal markers. Original magnification is 400× for all photos except **(B)**, **(E)**, **(K)**, and **(L)**, which are 100×. **A**: GST pi IHC of normal prostate showing intense staining of normal basal cells (positive control); (B) GST pi IHC of hormone naïve prostate cancer (negative control) with normal gland as internal positive control (top center); **(C)** GST pi IHC of hormone independent prostate cancer showing staining in stromal cells; **(D)** another example of GST pi IHC of hormone independent prostate cancer with staining in stromal cells; (E) intravascular prostate cancer lung metastatasis (top center) negative for GST pi by IHC surrounded by GST pi positive normal lung; **(F)** "Channel TURP" specimen from a patient with hormone independent prostate cancer positive for GST pi in stromal cells but not cancer cells; **(G)** another "channel TURP" specimen from a patient with hormone independent prostate cancer positive for GST pi in stromal cells; **(H)** vimentin staining of an adjacent section of the same channel TURP specimen shown in (G). Note that the pattern of vimentin staining closely resembles GST pi staining, suggesting that GST pi is expressed in reactive fibroblasts and/or myofibroblasts; **(I)** smooth muscle alpha actin staining of an adjacent section from the same channel TURP specimen shown in (G). Note that the staining pattern is very different from GST pi staining pattern, suggesting that GST pi is not expressed in fully differentiated smooth muscle cells; **(J)** calponin staining of an adjacent section from the same channel TURP specimen shown in (G); note that the staining pattern is different than that for GST pi, again suggesting that GST pi is not expressed in differentiated smooth muscle cells; (K) smooth muscle alpha actin staining of smooth muscle bundles (positive control) from the same channel TURP specimen shown in (G); (L) vimentin staining of a section adjacent to that shown in (K) showing staining only between muscle bundles; **(M)** GST pi immunofluorescence analysis of normal prostate showing intense staining in basal cells; **(N)** vimentin immunofluorescence analysis of the same section shown in (M) with staining primarily in stromal cells; **(O)** convergence of images (M) and (N) showing GST pi co-localization in only a few normal stromal cells.

(Fig. 1B, Table I). We next examined lymph nodes from patients found to have nodal metastases during lymphadenectomy prior to planned radical prostatectomy. GST pi was detectable in only one of the seven specimens, suggesting that re-expression of GST pi is not a common feature of progression to metastatic cancer (Table I).

Previous studies showed that several hormone independent human prostate cancer cell lines express high levels of GST pi, whereas a hormone dependent cell line, LNCaP, does not [13]. We therefore hypothesized that GST pi re-expression by cancer cells is a common feature of progression to hormone independence. To test this hypothesis, we examined GST pi expression and distribution in autopsy specimens from patients who died of hormone independent prostate cancer. GST pi was detectable in cancer cells in only two of seven metastases, one of which also stained positive in the stroma (Table I). GST pi expression in prostate cancer cell lines as shown previously [13] may therefore partially reflect the high oxidative stress of cell culture conditions rather than hormone independence, as originally hypothesized. In contrast to rare expression of GST pi in prostate cancer cells, GST pi was present in the stromal compartment of 8 of 12 hormone independent tumors obtained by autopsy (Fig. 1C,D, Table I). No staining was detected in control sections treated without primary antibody, confirming the specificity of the results (not shown). In one of the four GST pi negative autopsy specimens from patients with hormone independent cancer, a bone metastasis, GST pi was expressed in cancer cells only (Table I). In the remaining three, GST pi was abundant in normal tissue surrounding metastatic foci, consistent with previous studies showing that GST pi is expressed in normal lung and adrenal (Fig. 1E) [19,20]. GST pi was therefore expressed in or around every one of the 14 metastatic lesions examined, most commonly within the stromal compartment.

To further test the link between hormone independence and stromal GST pi expression, we examined nine transurethral resection ("channel TURP") specimens from patients who underwent the procedure for palliation of progressive hormone independent cancer. GST pi was detectable in the stroma of all nine specimens (Fig. 1F, Table I) but was undetectable in cancer cells, further suggesting that GST pi expression in the stromal compartment is a common feature of hormone independent prostate cancer, rather than metastatic cancer per se. GST pi expression within hormone independent tumors was confirmed by Western blot analysis of one such cancer obtained by TURP (Fig. 2).

Precise quantitation of staining intensity is not possible with immunohistochemistry. However, we subjectively graded specimens that were positive for stromal GST pi on a 1–4 scale, with 4 corresponding to staining intensity found in normal basal cells, and 1 corresponding to faint staining (Table I). We also measured GST pi staining intensity in two representative specimens semi-quantitatively using image analysis software (see Materials and Methods). Based on this analysis, GST pi protein was approximately seven times more abundant per cell in normal basal cells than it was in stromal cells within a typical channel TURP specimen.

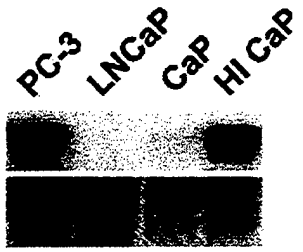

Fig. 2. Western blot analysis of GST pi levels in human prostate cancer specimens. PC-3 is a hormone independent cell line, whereas LNCaP is a hormone dependent cell line. CaP is a radical prostatectomy specimen taken from a hormone naïve patient, whereas HI CaP is a transurethral resection ("channel TURP") specimen from a patient with hormone independent cancer. Ponseau S staining is shown in the lower panel as a protein loading control.

Cellular Localization of GST pi Expression in Stroma of Hormone Independent Prostate Cancer

The human prostate gland stroma is composed primarily of smooth muscle cells and fibroblasts. Most human carcinomas are associated with some type of stromal compartment reaction, which is typified by an overaccumulation of extracellular matrix and myofibroblasts adjacent to carcinoma cells [21]. Myofibroblasts have characteristics of both smooth muscle cells and fibroblasts. In the current studies, stromal GST pi in hormone independent prostate tumors was confined to cells situated between bundles of thin cells with linear nuclei that morphologically resembled smooth muscle cells (Figs. 1C,G). We therefore hypothesized that smooth muscle cells in the stroma do not express GST pi, whereas fibroblasts and/or myofibroblasts do. To test this hypothesis, we performed immunhistochemical analysis of hormone independent prostate cancer specimens with antibodies to vimentin, smooth muscle α-actin, and calponin. Vimentin is an intermediate filament that is a marker of mesenchymal cells, whereas α-actin and calponin are smooth muscle markers. α-actin is expressed during the early stages of smooth muscle differentiation, and calponin is

expressed in fully differentiated cells. One antibody at a time was used to stain adjacent sections. We found that the distribution of GST pi staining (Fig. 1G) most closely coincided with vimentin staining (Fig. 1H), whereas smooth muscle α-actin staining (Fig. 1I) and calponin staining (Fig. 1J) coincided with the thin, GST pi-negative cells mentioned above. As a control, muscle bundles not associated with cancer stained with antibody to smooth muscle actin (Fig. 1K) but not with antibody to vimentin (Fig. 1L), confirming the specificity of results depicted in Figure 1H–J. These results suggested that GST pi is expressed by fibroblasts and/or myofibroblasts but not by fully differentiated smooth muscle cells in the stroma of hormone independent prostate cancer.

Immunofluorescence Microscopy of GST pi Expression in Hormone Independent Prostate Cancer

We next used immunofluorescence microscopy for co-localization studies to confirm the identity of the GST pi-expressing stromal cells in hormone independent prostate cancer. As a control, we first double stained sections of normal prostate for GST pi and vimentin. Some background signal due to auto-fluorescence and nonspecific antibody binding was observed (not shown). Nonetheless, specific GST pi signal was easily detectable above background in basal cells (Fig. 1M), and specific vimentin signal was easily detectable above background in the stromal compartment of normal tissue (Fig. 1N). Interestingly, a few stromal cells clearly stained intensely with both antibodies (Fig. 1O), suggesting that some normal stromal cells express GST pi. In contrast to normal prostate, tumors had high levels of auto-fluorescence, which probably originated from collagen, flavin coenzymes (FAD and FMN), and reduced pyridine nucleotides (e.g., NADH) [22]. Pretreatment of sections with collagenase [23] or sodium borohydride [24], as described previously, did not reduce autofluorescence sufficiently to allow specific identification of GST pi expressing cells in tumors. Unacceptably low signal relative to background was also probably attributable to relatively low stromal cell GST pi expression as compared to normal basal cells, as noted above. Immunofluorescence microscopy therefore did not allow for definitive localization of GST pi in tumor sections, despite the fact that GST pi was readily detectable by immunohistochemistry.

DISCUSSION

The current studies are the first to focus specifically on GST pi expression in the stromal compartment of prostate tumors. The stroma was once viewed as a passive support structure that contributes little to the overall biological function of tissues. However, it is now understood that the stroma is a dynamic environment that directly influences epithelial cell behavior and performs tissue repair in response to injury [25]. The stromal compartment is a complex arrangement of stromal cells and extracellular matrix plus associated growth factors, regulatory molecules, and remodeling enzymes. Blood vessels, nerves, and immune cells are also integral parts of the stroma. These components act in a coordinated manner to regulate cell function and maintain overall tissue homeostasis [25].

In addition to transformed carcinoma cells, tumors also contain stromal cells, extracellular matrix, newly formed blood vessels, and immune components. However, there is considerable evidence that tumor stroma differs from normal stroma. The stromal compartment is thought to maintain tissue homeostasis by reacting to carcinoma in a process similar to the general wound repair pathway [25]. The result of this response is the creation of a new stromal microenvironment, into which carcinoma grows and invades. Studies indicate that this new reactive stroma environment enhances tumorigenesis by supporting cancer cell survival, proliferation and migration, and by inducing angiogenesis. Therefore, it is becoming clearer that cancer progression is not exclusively regulated by the disruption of oncogene and tumor suppressor pathways in cancer cells. Much evidence now suggests that cancer progression also depends on the stromal compartment to create a more tumor promoting microenvironment [25].

Previous studies showed that GST pi expression is lost in prostate cancer cells as a result of GST pi CpG island hypermethylation and resultant transcriptional silencing [13]. GST pi was incidentally found in prostate tumor stroma in those studies, but the finding and its possible implications were not discussed [13,14]. The current studies show that GST pi expression by stromal cells is a common feature of hormone independent tumors. In the few cases that lacked stromal GST pi, GST pi was detectable in cancer cells and/or normal surrounding tissue. These results strongly suggest that GST pi plays important roles in prostate cancer progression despite the fact that it is rarely expressed by prostate cancer cells themselves in hormone naïve patients. In a previous study, neoadjuvant hormonal deprivation for 3 months before radical prostatectomy did not induce GST pi expression in prostate cancer or stromal cells [26]. Stromal GST pi expression therefore appears not to be a reflection of hormonal treatment but rather of hormonal independence.

Several previous studies showed that most human tumors and cancer cell lines contain increased GST pi

levels relative to their tissues of origin [2,7,8] and that GST pi expression in many tumors is inversely correlated with prognosis and response to chemotherapy [9]. GST pi is therefore thought to contribute to the multidrug-resistant phenotype observed in cancer [1,9–12]. Evidence to date therefore suggests a new paradigm for GST pi in prostate cancer. Abundant GST pi in normal prostate basal cells apparently protects glandular epithelium from carcinogens in order to maintain tissue homeostasis. GST pi gene inactivation during early stages of carcinogenesis may render prostate epithelial cells susceptible to genomic alterations by electrophilic or oxidant carcinogens that result in a selective growth advantage [27]. GST pi expression remains low in tumors until development of hormone independence, at which point cancer cells induce an injury response in the stroma which results in GST pi expression. This injury response may result from secretion of paracrine factors by tumors and may at least partially account for chemoresistance of hormone independent prostate cancer in light of GST pi's role as a detoxifying enzyme. Increased expression of GST pi in the stroma of hormone independent tumors may result from recruitment or proliferation of stromal cells that normally express GST pi or re-expression of GST pi in cells in which the gene is usually transcriptionally silent. Future studies will be required to test these possibilities. In light of the importance of tumor stroma during cancer progression, stromal GST pi may have promise as a therapeutic target in the future [28].

CONCLUSIONS

The current results suggest that prostate cancer cells induce an injury response in the stroma during progression to hormone independence which results in GST pi expression. Stromal GST pi may contribute to chemoresistence of advanced prostate cancer.

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Methionine restriction selectively targets thymidylate synthase in prostate cancer cells

Shan Lu[a], George L. Chen[a], Chengxi Ren[a], Bernard Kwabi-Addo[b], Daniel E. Epner[a,*]

[a]Department of Medicine, VA Medical Center, Baylor College of Medicine, Medical Service (111H), 2002 Holcombe Blvd., Houston, TX 77030, USA
[b]Department of Pathology, VA Medical Center, Baylor College of Medicine, Houston, TX 77030, USA

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Abstract

Tumor cells are more sensitive to methionine restriction than normal tissues, a phenomenon known as methionine auxotrophy. Previous studies showed that 5-fluorouracil and methionine restriction act synergistically against a variety of tumors. The purpose of the current studies was to determine the molecular mechanism(s) underlying this synergy. 5-Fluorouracil is known to inhibit thymidylate synthase (TS), a key enzyme that transfers a methyl group from 5,10-methylene-tetrahydrofolate to dUMP during nucleotide biosynthesis. We found that methionine restriction reduced 5,10-methylene-tetrahydrofolate levels by 75% and selectively inhibited TS activity in PC-3 human prostate cancer cells within 24 hr, whereas it did not in normal prostate epithelial cells. The observed fall in TS activity was accompanied by a commensurate reduction in TS protein levels as determined by western blot analysis. In contrast, 5-fluorouracil inhibited TS activity by >90% but increased TS protein levels. This increase was abrogated by methionine restriction. Surprisingly, methionine restriction increased ^{3}H-leucine incorporation in PC-3 cells over the first 24 hr, suggesting that reduction of TS levels was not simply due to global protein synthesis inhibition. Methionine restriction also significantly reduced the ratio of dUMP to dTTP in PC-3 cells, creating an imbalanced nucleotide pool. These results suggest that synergy between methionine restriction and 5-fluorouracil is attributable to multiple factors, including depletion of reduced folates, selective inhibition of TS, and creation of an imbalanced nucleotide pool. Dietary and/or enzymatic methionine restriction combined with 5-fluorouracil has great promise as a novel treatment for advanced cancer.

Keywords: Methionine; Folate; Thymidylate synthase; Prostate neoplasms

1. Introduction

Methionine is an essential amino acid that cannot be synthesized from any of the other standard amino acids. Nonetheless, normal mammalian cells proliferate normally in the absence of methionine as long as homocysteine is present in the growth medium [1], and animals fed diets in which methionine has been replaced by homocysteine suffer no ill effects and grow normally [2,3]. Homocysteine is a nonstandard amino acid that has the same structure as methionine except that it lacks the methyl group (Fig. 1). Methionine independence of normal tissues is due to remethylation of homocysteine to methionine by the enzymes 5-methyltetrahydrofolate homocysteine methyltransferase and betaine-homocysteine methyltransferase. Although these enzymes are functional in some tumors [4], most tumors are dependent upon exogenous, preformed methionine and therefore fail to grow even in the presence of homocysteine [5–8]. Dietary methionine restriction causes regression of animal tumors, including human prostate cancer xenografts in nude mice [9,10] and inhibits metastasis in animal models [3,11]. Methioninase, an enzyme that degrades methionine and homocysteine, also inhibits growth of solid tumors and leukemia in animals [12–17]. One clinical trial of chemotherapy combined with short-term methionine restriction by total parenteral nutrition showed preliminary evidence of activity against gastric cancer [18]. In addition, a recent clinical trial showed that dietary methionine restriction is safe and feasible in adults with metastatic cancer, and results in significant reduction of plasma methionine levels [19].

The selective antitumor activity of methionine restriction is not due to an *absolute* difference between benign

* Corresponding author. Tel.: +1-713-794-7980; fax: +1-713-794-7938.
E-mail address: depner@bcm.tmc.edu (D.E. Epner).

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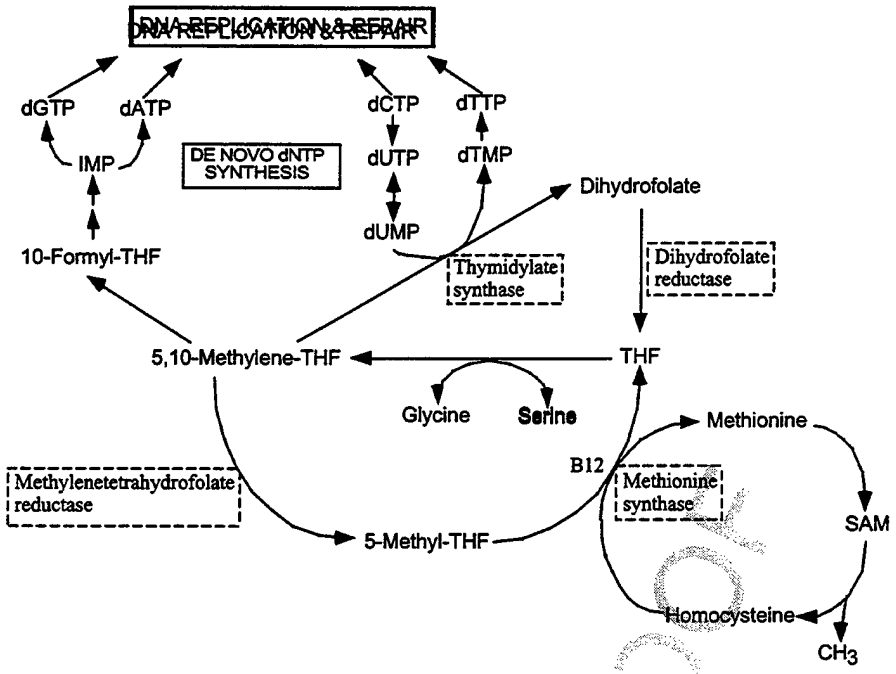

Fig. 1. Schematic overview of folate-derived one carbon (methyl) metabolism in nucleotide and methionine biosynthesis.

and malignant tissues, since neither can survive for long in the *complete* absence of methionine. Rather, tumors are *relatively* more sensitive to methionine restriction than normal tissues are, just as many tumors are relatively more sensitive to chemotherapy and radiation therapy. In contrast, restriction of other essential amino acids is either very toxic or ineffective [20]. Methionine restriction therefore does not represent indiscriminate "starvation".

The molecular mechanisms underlying methionine dependence of cancer cells have not been fully elucidated, but they probably relate to one or more of the specialized functions of methionine that distinguish it from other amino acids. Methionine is the immediate precursor of *S*-adenosylmethionine (SAM), the major methyl donor for methylation of DNA, RNA, and other molecules (Fig. 1). Other investigators have suggested that methionine dependence of tumors is due to elevated rates of transmethylation in cancer cells compared to corresponding normal tissues [21,22].

Folate serves as a bridging molecule for nucleotide and methionine biosyntheses, since folate-derived methyl groups are required for biosynthesis of methionine and nucleotides. In normal tissues, such as liver and kidney [23], methionine restriction and concomitant reduction of SAM diverts methyl groups from 5-methyl-tetrahydrofolate to methionine biosynthesis, thereby leading to irreversible conversion of 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate (Fig. 1) [24]. This process is referred to as the "methyl folate trap" [25]. This diversion of folate to methionine synthesis reduces normal *de novo* nucleotide biosynthesis, since folate-derived methyl groups are essential for both purine and pyrimidine synthesis. Thymidylate synthase (TS; EC 2.1.1.45) is an S-phase enzyme that catalyzes the reductive methylation of dUMP by 5,10-methylene-tetrahydrofolate, generating dTMP and dihydrofolate [26]. Folate deficiency causes accumulation of dUMP, which incorporates into DNA instead of thymine [27–29]. This leads to excessive uracil incorporation into DNA, which causes point mutations, single- and double-stranded DNA breaks, micronucleus formation, and DNA hypomethylation.

TS is an attractive target for anticancer drug design in light of its critical role in dTMP synthesis. Inhibitors of TS, such as the fluoropyrimidines 5-FU and 5′-fluoro-2′-deoxyuridine (FdUrd) and the antifolate methotrexate are widely used as cancer chemotherapy agents [30,31]. Newer antifolates, such as nolatrexed (AG337, Thymitaq), pemetrexed (LY231514), raltitrexed (Tomudex), are under development [31]. In growing cells, fluoropyrimidines (e.g. 5-FU and FdUrd) are metabolized to 5′-fluoro-2′-deoxyuridylic acid (FdUMP), which inhibit TS via formation of a covalent complex containing the nucleotide analog, 5,10-methylene-tetrahydrofolate, and TS [32]. This ternary complex is quite stable, resulting in prolonged inhibition of the enzyme and depletion of dTMP pools. The resulting imbalanced nucleotide supply compromises DNA replication, resulting in DNA strand breaks and apoptotic cell death, which in this context is referred to as "thymineless death" [33,34].

Clinical efficacy of 5-fluorouracil is enhanced by pretreatment of patients with folinic acid [35,36]. Folinic acid enhances the inhibitory effect of 5-fluorouracil on TS by increasing levels of 5,10-methylene-tetrahydrofolate, thereby stabilizing the complex between chemotherapy

drug and enzyme. The multi-step process of converting folinic acid to 5,10-methylene-tetrahydrofolate is coupled to methylation of homocysteine to form methionine. The efficacy of folinic acid therefore depends upon *de novo* methionine synthesis. Methionine deprivation would therefore be expected to accelerate conversion of folinic acid to 5,10-methylene-tetrahydrofolate by increasing conversion of homocysteine to methionine [23,37–39].

Based on the above considerations, Machover *et al.* [37] and Mini *et al.* [40] hypothesized that methionine depletion would increase the rate at which folinic acid is converted to 5,10-methylene-tetrahydrofolate in leukemia cells in culture, thereby enhancing the efficacy of folinic acid and 5-fluorouracil. In studies by Machover *et al.*, methionine depletion was accomplished with recombinant methioninase, an enzyme that cleaves methionine. Counter to their hypothesis, however, 5,10-methylene-tetrahydrofolate and tetrahydrofolate levels did not increase in those studies [37]. Nonetheless, methionine depletion did enhance the efficacy of folinic acid combined with 5-fluorouracil, which was attributed to decreased TS activity rather than the expected increase in 5,10-methylene-tetrahydrofolate [37].

Multiple cell culture and animal studies, including those by Machover *et al.* [37] also showed synergy between dietary and/or enzymatic methionine restriction combined with 5-fluorouracil even in the *absence* of folinic acid [16,41,42]. However, the mechanisms underlying this observed synergy remain unclear. Based on the "methyl *folate* trap" argument outlined above, one would expect 5,10-methylene-tetrahydrofolate levels to become *depleted* rather than enhanced by methionine restriction in the *absence* of a source of exogenous folate, namely folinic acid. Consistent with that possibility, Machover *et al.* [37] found that the combined level of 5,10-methylene-tetrahydrofolate plus tetrahydrofolate fell by approximately 50% in leukemia cells in culture in response to partial methionine depletion. The observed reduction was attributable to a 60–70% reduction of tetrahydrofolate, since 5,10-methylene-tetrahydrofolate levels appeared to be unaffected.

We undertook the current studies to test the hypothesis that deprivation of methionine in the absence of folinic acid does in fact reduce intracellular folate levels in methionine-dependent prostate cancer cells, resulting in an imbalanced nucleotide pool. We also determined whether methionine restriction affected levels or enzymatic activity of TS. We found that methionine restriction affected multiple aspects of folate and nucleotide metabolism.

2. Materials and methods

2.1. Cell culture

Human prostate cancer PC-3 cells (American Type Culture Collection) were maintained in RPMI-1640 (Life Technologies, Inc.) supplemented with 10% FBS (HyClone Laboratories) at 37° in 5% CO_2. Methionine restriction experiments were performed in methionine-free RPMI-1640 (Life Technologies, Inc.) supplemented with 10% FBS and 100 μM homocysteine (Sigma Chemical Co.). Folate restriction experiments were performed in folate-free RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% FBS. Primary culture prostate epithelial cells (PrEC) were purchased from BioWhittaker, Inc. and Clonetics Products. PrEC was maintained in the prostate epithelial basal medium and methionine restriction was performed in prostate epithelial cell labeling medium without methionine supplemented with 100 μM homocysteine, which were obtained from the company.

2.2. Reagents

Antibody for TS was obtained from Lab Vision Corporation. 5,10-Methylene-tetrahydrofolate was purchased from Schircks Laboratories. [5-^{3}H]-dUMP was from Amersham (Amersham Co.).

2.3. Western blot analysis

Aliquots of samples with 50 μg of protein, determined by the Bradford assay (BioRad), were mixed with loading buffer (final concentrations of 62.5 mM Tris–HCl (pH 6.8), 2.3% SDS, 100 mM dithiothreitol, and 0.005% bromophenol blue), boiled, fractionated in a 10% SDS–PAGE, and transferred onto a 0.45-μm nitrocellulose membrane by electroblotting (BioRad). The membranes were blocked with 2% fat-free milk in PBS, and then probed with first antibody (0.05 μg/mL IgG) in PBS containing 0.1% Tween 20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horseradish peroxidase-conjugated $F(ab')_2$ of secondary antibody (BioRad) in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL western blotting detection system.

2.4. Thymidylate synthase assay

TS assay was performed as previously described [43]. Briefly, 25 μL of cell extract containing 50 μg protein, 5 μL 6.5 mM 5,10-methylene-tetrahydrofolate, and 10 μL of Tris–HCl buffer were combined at room temperature. The assay was initiated by addition of 10 μL [5-^{3}H]-dUMP 1 uM (1.0 mCi/mL, Amersham Pharmacia Biotech), incubated for 30 min at 37°, and stopped by addition of 50 μL ice-cold 35% trichloroacetic acid and 250 μL of 10% neutral activated charcoal. After centrifugation, 150 μL of the supernatant were counted by liquid scintillation. TS activity was proportional to the amount of tritium released from [5-^{3}H]-dUMP into solvent upon dTMP formation.

2.5. Primer design and synthesis for TS quantitative real-time PCR

Oligonucleotide primers for TS were designed using Molecular Beacon program (PREMIER Biosoft International). Primers were sense: 5′-GCAGATCCAACACATCCTC-3′; and antisense: 5′-AAACACCCTTCCAGAACAC-3′. The nucleotide position for the amplification product as given by the GenBank accession number (AB077208) is 105–253. Oligonucleotide primers for β-actin were designed using Baylor College of Medicine Primer Selection program (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html). Primers were sense: 5′-AGCACGGCATCGTCACCAACT-3′; and antisense: 5′-TGGCTGGGGTGTTGAAGGTCT-3′. The nucleotide position for the amplification product as given by the GenBank accession number (X00351) is 256–435. Primers were carefully designed to cross exon/intron regions, avoid the formation of primer–dimer, hair pin and self-complementarity. Synthetic oligonucleotide primers were obtained from Invitrogen (Life Technologies).

2.6. cDNA synthesis and quantitative real-time PCR

Total RNA (5 μg) was treated with DNase1 (Invitrogen) and incubated at 70° for 10 min. The RNA was then reverse-transcribed in the presence of 10 mM dithiothreitol (DTT), 50 ng of random hexamers, 0.25 mM each of the four deoxytriphosphate nucleotides and 200 U of Superscript™ II Reverse Transcriptase in a total volume of 20 μL according to the manufacturer's protocol (Invitrogen). Residual RNA was removed by adding 1 μL of *Escherichia coli* RNase H (Invitrogen; 222 U/μL) and the reaction incubated at 37° for 20 min. Quantitative PCR was carried out by adding 5 μL of template cDNA to a final 25 μL reaction volume containing 3 mM $MgCl_2$; 0.4 μM each forward and reverse primers and 2.5 μL of LC-FastStart DNA Master SyBr Green 1 (Roche). Real-time PCR was done using the iCycler iQ instrument (BioRad Laboratories) using optimized PCR reaction conditions. Amplification of TS and β-actin was carried out as follows: a 3 min hot start at 95°, followed by 40 cycles of denaturation at 95° for 30 s, annealing at 56° for 20 s and a 72° extension for 30 s. Each assay included a negative control and the experiment was done in duplicate. The fluorescence emitted by the reporter (SyBr Green) dye was detected online in real-time, and the threshold cycle (C_t) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The C_t value is the fractional cycle number at which the fluorescence generated by the reporter dye exceeded a fixed level above baseline. The TS signal was normalized against the relative quantity of β-actin and expressed as $\Delta C_t = (C_{tTS} - C_{t\beta\text{-actin}})$. The change in TS signal relative to the reference signal (one sample) was expressed as $\Delta\Delta C_t = (\Delta C_{tcontrol} - \Delta C_{tsample})$. Relative changes in expression was then calculated as $2^{[-\Delta\Delta C_t]}$.

2.7. 5,10-Methylene tetrahydrofolate assay

Intracellular 5,10-methylene-tetrahydrofolate was measured by the standard TS assay as described above. Fifty micrograms control PC-3 cell extract was used as the source of TS for each assay. Folate extracts from four million cells in 100 μL (containing unknown amounts of 5,10-methylene-tetrahydrofolate) were added to the standard reaction mixture. Release of tritium into the solvent in this assay therefore reflected 5,10-methylene-tetrahydrofolate levels rather than TS activity. Folate extraction was performed as previously described [37]. Briefly, cells were suspended in cold buffer (50 mM Tris–HCl (pH 7.4), 50 mM sodium ascorbate, and 1 mM EDTA) to a density of 4×10^7 cells/mL. Cells were lysed in a boiling water bath for 3 min and centrifuged at 14,000 *g* for 5 min at 4°. The supernatant was used immediately for the assay or frozen at −70° until used.

2.8. Measurement of total cellular protein synthesis

2×10^5 of PC-3 cells were seeded per well in 6-well plates. The next day, 3 mL of either complete medium or methionine-free medium containing 1 μL of ^{3}H-leucine (1.0 mCi/mL, Amersham Pharmacia Biotech) were added into each well at 1, 3, 6, 24 hr before harvest of the total cellular protein. The cells were then washed with PBS twice and lysed in 100 μL of lysis buffer (20 mM Tris–HCl, pH 8.0; 137 mM NaCl; 10%, w/v glycerol; 10 mM NaF; 1% Triton X-100; 1 mM Na_3VO_4; 2 mM EDTA; 1 mM PMSF; 20 μM leupeptin; and 0.15 U/mL aprotinin). The total cellular protein was then concentrated by TCA precipitation. The samples containing 10% TCA were incubated on ice for 30 min and spun at 14,000 *g* for 5 min. The precipitated protein was dissolved in 50 mL of 0.1 M NaOH. Radioactivity was determined by liquid scintilation counter.

2.9. Preparation of cellular dNTP extract for HPLC analysis [44]

5×10^5 cells from each sample were mixed with 10 μL 0.6 M trichloroacetic acid. The lysate was incubated at 4° for 30 min. After centrifugation, the acidic supernatant was transferred to a microcentrifuge tube. An equal volume of ice cold 80% 1,1,2-trichlorotrifluoroethane and 20% tri-*n*-octylamine was added to the lysate. The mixture was vortexed for 15 s and then centrifuged at 14,000 *g* for 5 min at 4°. The aqueous supernatant was removed and centrifuged at 14,000 *g* for 5 min at 4°. Samples were stored at −70° until used.

2.10. HPLC analysis

Chromatographic analyses were performed with a Waters 625 LC System (Waters Corporation) consisting of a Waters 625 Fluid Handling Unit with a Rheodyne 9125-080 Manual Injector and 20 μL sample loop, 625E Powerline Controller, and 484 Tunable UV Detector. Component separation was achieved using a reversed phase SS Exsil ODS column (5 μM particle size, 4.6 mm × 250 mm, SGE Incorporated). The column was maintained at ambient temperatures. The methodology of Cross *et al.* [44] with some modification was used to separate the nucleotides. Briefly, two buffers comprised the mobile phase—Buffer A consisting of 0.2 M $(NH_4)H_2PO_4$ in 1.0 M KCl at pH 5.35, and Buffer B consisting of 0.2 M $(NH_4)H_2PO_4$ in 1.25 M KCl and 10% methanol at pH 5.0. pH was adjusted with NaOH solution and Buffer B was titrated after the addition of methanol. UV detection was at 250 nm. Solvent flow rate was maintained at 0.8 mL/min during the elution gradients. The elution gradients were as follows: 100% Buffer A for 8 min followed by a 13 min linear gradient to 75% Buffer A and 25% Buffer B. At 22 min, a 2 min linear gradient to 15% Buffer A and 85% Buffer B started. 15% Buffer A and 85% Buffer B was maintained until the end of the run at 40 min. Afterwards, the column was regenerated with 100% Buffer A at 1.0 mL/min for 15 min.

A series of standards containing varying amounts of dUMP and dTTP ranging from 1.0 to 0.02 nmol was analyzed using the above methodology. The different quantities and their correlating absorption areas existed in a linear relationship. Using the least squares method, a linear equation was generated. This linear equation was used to calculate the quantity of dUMP or dTTP represented by the absorption peaks in the chromatograms generated from our experimental samples.

2.11. Cell growth assay

Tumor cell growth was estimated by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as previously described [45]. Briefly, PC-3 cells were harvested by exposure to 0.25% trypsin/0.02% EDTA (w/v) and seeded into 96-well microculture plates at a density of 2500 cells/well in RPMI 1640 medium supplemented with 10% FBS. After incubation in 5% CO_2 at 37° overnight, the cells were incubated with fresh medium containing either 0, 10, or 100 μM methionine with or without 5-FU for 3 days. Thereafter, 20 μL of MTT (2.5 mg/mL in phosphate-buffered saline, PBS) was added to each well, and the cells were further incubated for 2 hr at 37° to allow complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable cells. After removal of residual dye and medium, 100 μL dimethylsulfoxide were added to each well, and the absorbance at 570 nm was measured with a microplate reader (BioRad).

3. Results

3.1. Methionine restriction reduced intracellular folate levels

We first measured intracellular 5,10-methylene-tetrahydrofolate levels in prostate cancer cells in order to investigate whether methionine restriction diverted folate to methionine synthesis, as it does in normal liver [24] and kidney cells [23] as a result of the "methyl folate trap" [25]. Methionine deprivation reduced the level of 5,10-methylene-tetrahydrofolate by 75% within 24 hr. The effect was maintained for up to 72 hr (Fig. 2A). As a control, we also measured intracellular 5,10-methylene-tetrahydrofolate levels in response to folate depletion for 24 hr. As expected, 5,10-methylene-tetrahydrofolate fell by 67% in PC-3 cells cultured in folate-free medium (Fig. 2B).

3.2. Methionine restriction inhibited TS activity

We next determined whether methionine restriction inhibited TS in prostate cancer cells, as it is known to

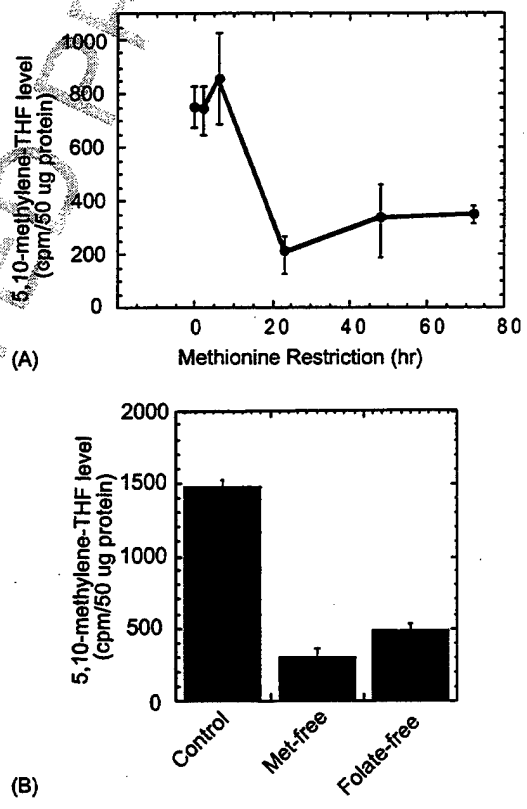

Fig. 2. Effect of methionine restriction on 5,10-methylene-tetrahydrofolate levels in PC-3 cells. (A) Kinetics of 5,10-methylene-tetrahydrofolate depletion in cells grown in methionine free medium for up to 72 hr. (B) 5,10-Methylene-tetrahydrofolate depletion in PC-3 cells after 24 hr in methionine free medium as compared to folate free medium. 5,10-Methylene-tetrahydrofolate levels were measured as described in Section 2. Values are mean ± SD, N = 5.

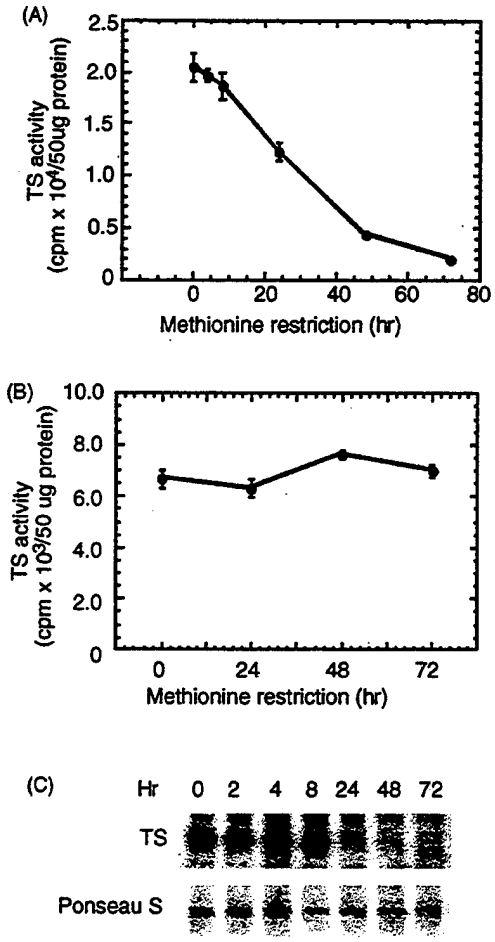

Fig. 3. Selective inhibition of TS activity in PC-3 cells by methionine restriction. TS activity in PC-3 cells (A) and normal prostate epithelial cells (B) grown in methionine free medium for up to 72 hr was measured as described in Section 2. (C) TS protein levels in PC-3 cells grown in methionine-free medium for up to 72 hr measured by western blot analysis as described in Section 2. Values in A and B are mean ± SD, N = 5.

in leukemia cells [37]. We found that methionine restriction inhibited TS activity in PC-3 cells by approximately 40% within 24 hr and by 80% in 48 hr (Fig. 3A). In contrast, TS activity in normal human prostate epithelial cells was unaffected by methionine restriction (Fig. 3B). The observed fall in TS activity in prostate cancer cells in response to methionine restriction was accompanied by a commensurate fall in TS protein levels by 80% within 24 hr. This 80% reduction was confirmed by western blot dilution experiments (not shown). TS protein was almost undetectable within 48 hr (Fig. 3C). TS RNA levels as measured by quantitative real-time PCR also fell by 74% within 24 hr and by 82% within 48 hr, as shown in Table 1.

In contrast to the observed fall in TS abundance, global cellular protein synthesis, as measured by tritiated leucine incorporation, was not significantly affected by methionine restriction within the first 24 hr (Fig. 4). In fact, leucine

Table 1
Quantitative RT–PCR of thymidylate synthase RNA levels in PC-3 cells in response to methionine restriction

	Sample				
	TS C_t value	β-Actin C_t value	ΔC_t	$\Delta\Delta C_t$	$2^{[-\Delta\Delta C_t]}$
Control	18.6	10.9	7.7	−2.85	7.21
24 hr	21.6	11.95	9.65	−0.9	1.86
48 hr	21.0	10.8	10.2	−0.35	1.27
72 hr	22.55	12.0	10.55	0.0	1.00

Levels relative to 72 hr of treatment are listed in the far right column. See Section 2 for experimental details.

incorporation during the first 6 hr of the experiment was actually greater in cells deprived of methionine than it was in control cells (Fig. 4).

As a control, we next measured TS enzymatic activity in PC-3 cells in response to 5-FU. As expected, 5-FU inhibited TS activity by 95% within 8 hr (Fig. 5A), whereas it dramatically *increased* TS protein level as determined by western blot (Fig. 5B). The observed TS protein accumulation in response to 5-FU was largely abrogated by concurrent methionine restriction (Fig. 5C).

3.3. Methionine restriction disrupted nucleotide balance

We next used HPLC to measure the effect of methionine restriction on nucleotide levels in PC-3 cells, since TS plays a central role in nucleotide biosynthesis. The ratio of dUMP to dTMP rose from 0.48 ± 0.07 at baseline (Fig. 6A and Table 2) to 1.75 ± 0.61 after 24 hr of methionine restriction (Fig. 6B and Table 2) and remained at about the same level for up to 48 hr (Table 2). 5-FU treatment was used as a positive control for TS inhibition, and, as expected, resulted in a dramatic increase in dUMP/dTTP ratio (Fig. 6C and Table 2).

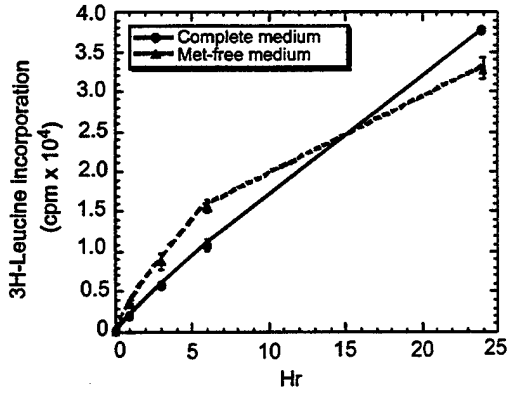

Fig. 4. Effect of methionine restriction on total cellular protein synthesis in PC-3 cells. Protein synthesis as determined by rate of ^{3}H-leucine incorporation was measured as described in Section 2. Values are mean ± SD, N = 5.

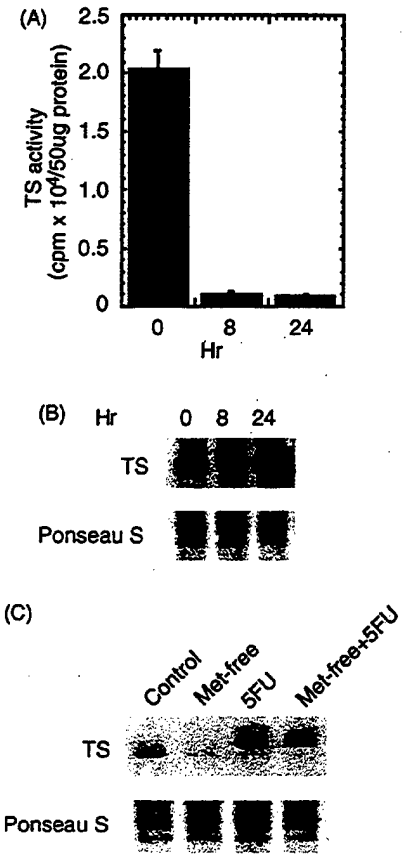

Fig. 5. Abrogation of TS up-regulation by methionine restriction in PC-3 cells following 5-FU treatment. TS activity (A) and abundance (B) following methionine restriction for up to 24 hr were measured with the standard TS enzyme assay and western blotting, respectively, as described in Section 2. (C) TS protein abundance under control conditions or after methionine restriction, 5 μM 5-FU, or methionine restriction + 5 μM 5-FU for 24 hr measured by western blot analysis. Values in A are mean ± SD, N = 5.

3.4. Methionine restriction enhanced PC-3 cells growth inhibition by 5-FU

Treatment of PC-3 cells with 5-FU alone (2.5 μM) for 3 days in medium containing 100 μM methionine inhibited growth by 25% as compared to control conditions (Fig. 7).

Table 2
Quantitative analysis of intracellular dUMP and dTTP level in response to methionine restriction in PC-3 cells by HPLC

	Treatment		
	dUMP[a]	dTTP[a]	dUMP/dTTP
Control	57.7 ± 18.1	117.0 ± 22.5	0.48 ± 0.07
Met-free—24 hr	145.4 ± 52.7	82.6 ± 2.3	1.75 ± 0.61
Met-free—48 hr	74.4 ± 15.7	40.6 ± 5.5	1.87 ± 0.56
5-FU—24 hr	1457.3 ± 265.9	191.7 ± 27.0	7.60 ± 0.99
5-FU—48 hr	867.3 ± 150.2	151.0 ± 30.6	5.75 ± 0.32

Data represent means ± SD. Experiments were repeated at least three times.

[a] pmol/500,000 cells.

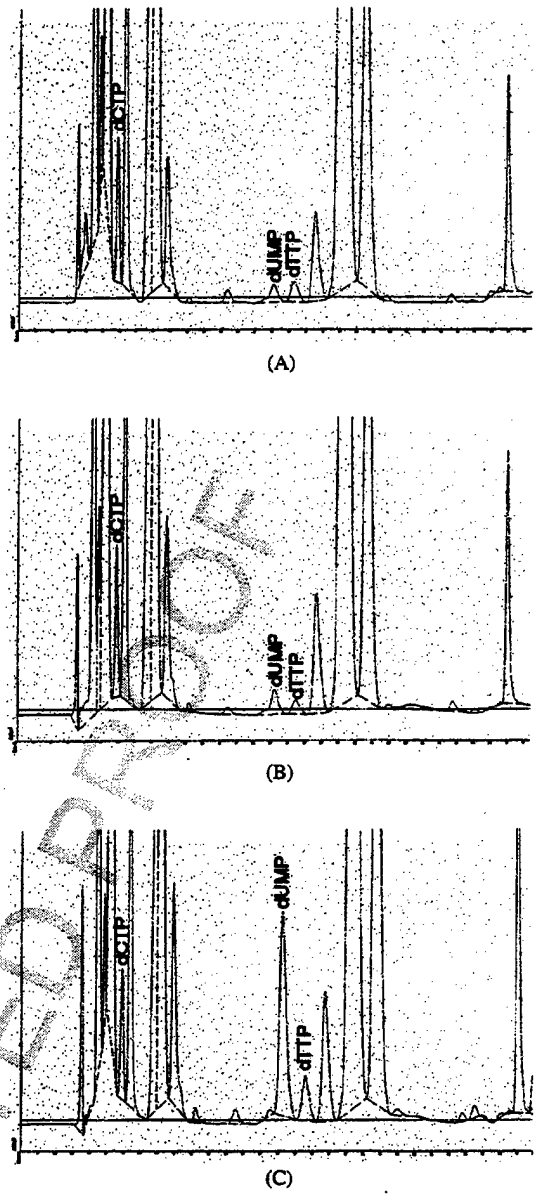

Fig. 6. Disruption of nucleotide balance in PC-3 cells in response to methionine restriction. HPLC nucleotide chromatograms under control conditions (A), following 24 hr of methionine restriction (B), or following 24 hr treatment with 5 μM 5-FU (C). Relevant nucleotide peaks are labeled.

Reduction of methionine levels in the medium to 10 μM in combination with 2.5 μM 5-FU inhibited growth by an additional 22% (57% reduction compared to control, Fig. 7). This level of depletion is achievable *in vivo* by dietary restriction in adults with metastatic cancer [19]. Further reduction of methionine in the medium combined with 5-FU inhibited growth by yet an additional 22% (total 79% growth reduction, Fig. 7). These highly restrictive conditions are also achievable *in vivo* in selected cancer patients treated with a restrictive diet alone and may be achievable in the majority of patients treated in the future with recombinant methioninase [46]. Results of these

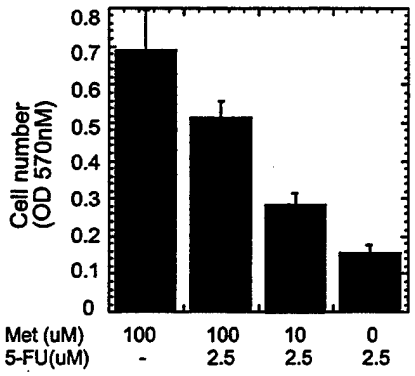

Fig. 7. Enhancement of growth inhibitory effects of 5-FU by methionine restriction. PC-3 cells were grown for 3 days under control conditions (100 μM lacking 5-FU), in the presence of 5-FU alone, or in the presence of 5-FU combined with moderate (10 μM) or severe (0 μM) methionine restriction. Cell number was determined by the MTT assay as described in Section 2. Values represent mean ± SD, N = 5.

growth inhibition studies were similar whether dialyzed serum or nondialyzed serum was used.

4. Discussion

The current results suggest that one mechanism by which methionine restriction induces prostate cancer cell cycle arrest and eventual apoptosis is by depleting 5,10-methylene-tetrahydrofolate, which is a critical precursor for nucleotide biosynthesis. Our data are consistent with those of Machover *et al.* [37], who also showed that methionine restriction reduced 5,10-methylene-tetrahydrofolate levels in cancer cells. In contrast, folinic acid, the formyl derivative of folic acid, potentiates the antitumor activity of 5-fluoruracil by *increasing* 5,10-methylene-tetrahydrofolate, thereby stabilizing the complex between 5-FU and TS. Our studies as well as previous ones [37] therefore suggest that synergy between 5-FU and methionine restriction is due at least in part to diversion of folate to methionine synthesis via the "methyl folate trap" [25].

We also found that methionine restriction inhibited TS activity in prostate cancer cells but not in normal prostate epithelial cells, which is a reflection of the greater methionine dependence of cancer cells relative to corresponding normal cells. TS inhibition in cancer cells was accompanied by a commensurate reduction in TS protein levels as measured by western blot analysis, suggesting that enzyme inhibition was largely, if not entirely, due to reduced enzyme abundance rather than enzyme inactivation. Surprisingly, global protein synthesis, as measured by ^{3}H-leucine incorporation, *increased* during the first several hours of methionine restriction, which is consistent with previous studies [47]. This result suggests that the observed fall in TS protein abundance was not simply due to global protein synthesis inhibition but rather to specific down-regulation of TS levels. This down-regulation was probably due to reduced TS mRNA levels, which fell in parallel with TS protein levels (Table 1). However, TS inhibition may also have been due to reduced folate levels in methionine-depleted cells, with consequent generation of ligand-free enzyme that additionally repressed TS mRNA translation or decreased stability of TS polypeptide.

The current results also suggest that synergy between methionine restriction and 5-FU is partially due to blockage of TS up-regulation, which is a major mechanism by which cancer cells become resistant to 5-FU [48,49]. TS inhibition subsequently leads to nucleotide imbalance, which is known to cause cells to undergo a form of apoptosis known as "thymineless death" [33,34].

Results of a phase I clinical trial of dietary methionine restriction for adults with advanced cancer from our institution indicated that dietary methionine restriction is safe and feasible for at least several weeks at a time and resulted in significant and clinically relevant declines in plasma methionine levels [19]. Nonetheless, it is probably unreasonable to expect patients to remain on a methionine restricted diet or any other severely restrictive diet for prolonged periods of time. Dietary methionine restriction will therefore most likely have the greatest impact when prescribed intermittently in combination with chemotherapy and/or recombinant methioninase, the methionine-degrading enzyme under development by Anticancer Incorporated and their pharmaceutical partners in Asia. In fact, sufficient pre-clinical data already exist to justify a clinical trial of dietary methionine restriction plus chemotherapy in selected tumor types, such as glioblastoma multiforme [12].

Acknowledgments

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